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Comparative Assessment of the Antioxidant Efficacy of *Trigonella foenum-graecum* and *Hibiscus rosa-sinensis* in Reversing Phenylhydrazine-Mediated Hemolytic Anemia in Mus Musculus

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Abstract: Investigation of antioxidant properties of *Trigonella foenum-graecum* (fenugreek) seed and *Hibiscus rosa-sinensis* (China rose) bark extracts in phenylhydrazine (PHZ)-induced anaemia models in mice. Ethanol extracts of both plant materials were prepared through maceration and evaluated for *in-vitro* antioxidant activity using DPPH radical scavenging, hydrogen peroxide scavenging, and reducing power assays. Results revealed dose-dependent antioxidant activity, with *Hibiscus rosa-sinensis* showing greater reducing power and *Trigonella foenum-graecum* demonstrating superior hydrogen peroxide and DPPH scavenging activity. *In vivo*, anaemia was induced in Swiss albino mice using PHZ (10 mg/kg body weight), leading to significant reductions in antioxidant enzyme levels including superoxide dismutase (SOD), reduced glutathione (GSH), and glutathione peroxidase (GSHPx). Treatment with individual and combined plant extracts significantly restored antioxidant parameters. Acute and sub-acute toxicity studies established the safety of both extracts up to 2000 mg/kg, and a combined dose of 400 mg/kg body weight was found effective and non-toxic. These findings support the therapeutic potential of *T. foenum-graecum* and *H. rosa-sinensis* as natural antioxidants with haemoprotective efficacy, particularly in oxidative stress-related anaemic conditions.

Keywords: *Trigonella foenum-graecum, Hibiscus rosa-sinensis, Antioxidant Activity, Phenylhydrazine, Anaemia, Mice Model.*

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I. INTRODUCTION

Anaemia is a widespread haematological condition marked by a decrease in red blood cell count or haemoglobin concentration, often resulting from oxidative stress-induced erythrocyte damage and impaired erythropoiesis (Thomas *et al*., 2013). Reactive oxygen species (ROS), such as superoxide anions and hydrogen peroxide, play a significant role in oxidative injury and haemolysis, contributing to the pathophysiology of anaemia (Jain *et al*., 1990; Ravi *et al*., 2004). In recent years, growing attention has been directed toward the use of medicinal plants with antioxidant properties to counteract oxidative damage.

Trigonella foenum-graecum (fenugreek) seeds and *Hibiscus rosa-sinensis* (China rose) bark are two such botanicals with reported antioxidant, anti-inflammatory, and hematinic effects. Fenugreek seeds are rich in flavonoids, polyphenols, and saponins, which contribute to their ability to scavenge free radicals and enhance endogenous antioxidant enzyme activity (Kaviarasan *et al*., 2007; Kumari & Jain, 2020). Similarly, *Hibiscus rosa-sinensis* has demonstrated potent antioxidant activity attributed to its phenolic and flavonoid constituents (Kumar *et al*., 2014; Kumari *et al*., 2020). These phytochemicals play a protective role against oxidative damage by modulating enzymatic and non-enzymatic antioxidant systems.

In vitro antioxidant models such as DPPH radical scavenging, reducing power assay, and hydrogen peroxide scavenging assays are widely used to evaluate the free radical neutralizing capacity of plant extracts (Bajpai *et al*., 2008; Ruch *et al*., 1989; Hemalatha *et al*., 2013). Meanwhile, in vivo models using phenylhydrazine (PHZ)-induced anaemia in mice provide a reliable experimental platform for assessing haemoprotective and antioxidant efficacy (Thomas *et al*., 2013). PHZ induces oxidative stress by generating ROS, resulting in erythrocyte lysis and oxidative damage to tissues,

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which can be mitigated by compounds that enhance the activity of antioxidant enzymes like superoxide dismutase (SOD), glutathione (GSH), and glutathione peroxidase (GSHPx) (Sinha *et al*., 2013; Jain *et al*., 1990).

This study investigates the antioxidant potential of ethanol extracts of *T. foenum-graecum* seeds and *H. rosa-sinensis* bark in PHZ-induced anaemic mice. The work includes comprehensive *in vitro* antioxidant assays, acute and sub-acute toxicity evaluations, and a detailed *in vivo* assessment of antioxidant enzyme parameters. The findings aim to elucidate the role of these extracts as potential natural therapeutic agents in the management of oxidative stress-induced anaemia.

II. MATERIALS AND METHODS

➢ *Collection of Plant Materials:*

1 kg each of *Trigonella foenum-graecum* seeds and *Hibiscus rosa-sinensis* bark were collected from the Sanjivni outlet of Vindhya Herbal, Bhopal.

- *Extraction of Plant Extracts:*

The bark of *Hibiscus rosa-sinensis* and seeds of *Trigonella foenum-graecum* were washed, and air-dried at room temperature. The dried material was coarsely powdered using a mechanical grinder. Extraction was carried out via maceration followed by Soxhlet using water as solvent for *Hibiscus* and Petroleum ether as solvent for *Trigonella* following the method described by Bajpai *et al.* (2008). The extract was filtered using Whatman No. 1 filter paper and concentrated using a rotary evaporator at reduced pressure. The dried extract was stored at 4°C until further use.

- *In-Vitro Antioxidant Activity:*

Reducing power method (RP), Hydrogen peroxide scavenging (H2O2) and DPPH scavenging activity were performed as following:

- *Reducing Power Assay:*

This test was performed according to Hemalatha *et al.* (2013). 800µl of the extract was mixed with 800µl of a 1% potassium ferricyanide [$K_3Fe(CN)_6$] and 400µl of phosphate buffer (0.2M, pH=6.6). The mixture was then incubated for 20 minutes at 50°C. 800µl (10%) Trichloroacetic acid (TCA) was added to the mixture, and centrifuged for 10 minutes at 3000r/t. Absorbance was taken after mixing 400µl of the supernatant solution with 400µl of distilled water and 80 µl $FeCl_3$ (0.1%) at 700nm. A higher reducing power was demonstrated by the reaction mixture's increased absorbance. The results were expressed as µg ascorbic acid equivalent/mg extract.

Calculation of % Reduction = [(Control Absorbance -Test absorbance) / Control Absorbance] X 100

- *Hydrogen Peroxide Scavenging (H2O2) Assay:*

Following the method of Ruch *et al.*, 1989 capacity to scavenge hydrogen peroxide was determine in sample extracts. 40 mM hydrogen peroxide solution is made in 50 mM pH 7.4 phosphate buffer. After adding extract (20-60µg/mL in distilled water) to hydrogen peroxide, absorbance at 230 nm was measured after 10 minutes and compared to a blank solution that contains phosphate buffer but without hydrogen peroxide. % scavenged (H_2O_2) was calculated as follows:

% scavenged (H2O2) = [(Ai – At) / Ai] x 100

Where Ai is the absorbance of control and At is the absorbance of test.

- *DPPH (2,2-Diphenyl-1-Picrylhydrazyl) scavenging activity (Pisoschi and Negulescu, 2011):*

An initial absorbance of 75 µl in 3 ml of methanol was obtained by preparing a stock solution (1.5 mg/ml in methanol). After 15 minutes, there was a decrease in absorbance when sample extract was present at various concentrations (10–100 µg/ml). A volume of 3 ml was created by adding methanol to 75 µl of DPPH solution. The absorbance was measured at 517 nm right away to obtain the control reading. Using methanol, the final volume was adjusted to 3 ml after 75 µl of DPPH and 50 µl of the test sample at various concentrations were placed in a series of volumetric flasks. Each of the three test samples underwent an identical processing step. The mean was finally applied. % % of antioxidant activity was calculated as follows:

% of antioxidant activity = [(Ai – At) / Ai] x 100

Where: Ai is the absorbance of control and At is the absorbance of test.

- *Animal Model and Ethical Approval:*

Swiss albino mice (*Mus musculus*), weighing 22–28g, were procured from Radharaman College of Pharmacy, Bhopal. The animals were maintained under standardized laboratory conditions (temperature: 22–28°C, relative humidity: 60–70%, 12-hour light/dark cycle) and provided a standard pellet diet (Sai Durga Feeds and Foods) and water ad libitum. All experiments were conducted at Xcellventure Institute of Fundamental Research Pvt. Ltd., Bhopal. Ethical approval was obtained from the IAEC, Radharaman College of Pharmacy, Bhopal (Reg. No. 1169/ac/08/CPCSEA).

- *Acute Toxicity Study:*

Swiss albino mice were divided into six groups (n = 6 per group). Group I served as the untreated control, while Groups II–VI received single oral doses of *Trigonella foenum-graecum* and *Hibiscus rosa-sinensis* extract at concentrations of 100, 500, 1000, 1500, and 2000 mg/kg body weight in distilled water. The control group received 150 µl of distilled water. The animals were monitored for 72 hours for toxic symptoms such as weakness, aggression, diarrhea, discharge from eyes/ears, noisy breathing, and mortality. The lethal dose (LD_{50}) was determined using the arithmetic method of Karbar (Aguiyi, 1996; Dede & Dogara, 2003).

- *Sub-Acute Toxicity Study:*

Mice were divided into six groups (n = 6 per group). Group I served as the control, receiving only 150 µl of distilled water, while Groups II–VI received daily oral doses

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of *Trigonella foenum-graecum* and *Hibiscus rosa-sinensis* extract at 100, 500, 1000, 1500, and 2000 mg/kg body weight for 21 days. Animals were monitored for signs of toxicity, including weakness, aggression, diarrhea, discharge from eyes/ears, noisy breathing, and mortality. The LD_{50} was calculated following the arithmetic method of Karbar.

Acute and sub-acute toxicity studies established the safety of the extract, determining non-toxic doses of 400 mg/Kg and 800 mg/Kg body weight each.

- *Induction of Anaemia and Study Plan:*

Phenylhydrazine (PHZ) was purchased from HiMedia Pvt. Ltd., Mumbai, and used to induce anaemia at a dose of 10 mg/kg body weight, following the protocol described by Thomas *et al*. (2013).

III. EXPERIMENTAL DESIGN

A total of 78 animals were used in the study and divided into the following experimental groups:

➢ Group I: (n = 30)

- Group I (A): Positive Control (n = 6)
- Group I (B): *Hibiscus rosa-sinensis* Dose 1 (400 mg/Kg b.wt) (no. = 6)
- Group I (C): *Hibiscus rosa-sinensis* Dose 2 (800 mg/Kg b.wt) (no. = 6)
- Group I (F): *Trigonella foenum-graecum* Dose 1 (400 mg/Kg b.wt) (n = 6)
- Group I (G): *Trigonella foenum-graecum* Dose 2 (800 mg/Kg b.wt) (n = 6)

➢ *Group II:*

Anaemia-Induced (n = 48) – Anaemia was induced by administering phenylhydrazine (PHZ) at a dose of 10 mg/kg body weight for 10 consecutive days (5 mg/kg body weight twice daily). Haematological parameters were recorded on Day 11 to confirm the induction of anaemia.

- Group II (A): Negative Control (Anaemia without treatment) (n = 6)
- Group II (D): Anaemia + *H. rosa-sinensis* Dose 1 (400 mg/Kg b.wt) (no.= 6)
- Group II (E): Anaemia + *H. rosa-sinensis* Dose 2 (800 mg/Kg b.wt) (no.= 6)
- Group II (H): Anaemia + *Trigonella foenum-graecum* Dose 1 (400 mg/Kg b.wt) (n = 6)
- Group II (I): Anaemia + *Trigonella foenum-graecum* Dose 2 (800 mg/Kg b.wt) (n = 6)
- Group II (J): Anaemia + *Trigonella foenum-graecum* + *Hibiscus rosa-sinensis* Dose of 400 mg/kg body weight (1:1) (n = 6)
- Group II (K): Anaemia + *Trigonella foenum-graecum* + *Hibiscus rosa-sinensis* Dose of 800 mg/kg body weight (1:1) (n = 6)
- Group II (S): Anaemia + Ferrous sulphate 0.0214 mg/kg b. wt. (n = 6)

For these experimental, Day 1 of treatment was considered the beginning of the study, including the negative control group, to assess the combined effects of *Trigonella foenum-graecum* and *Hibiscus rosa-sinensis* extract compared to standard drug ferrous sulphate on anaemia. Haematological parameters were recorded on Days 1, 15, 30, 45, and 60.

➢ *In Vivo Antioxidant Activity Assays*

- *Superoxide Dismutase (SOD):*

Assessed using a modified McCord and Fridovich (1969) method. Erythrocyte lysate (from 5% RBC suspension) was mixed with Tris–HCl buffer, EDTA, and pyrogallol. Absorbance at 420 nm was recorded over 3 minutes. One unit of SOD activity corresponds to 50% inhibition of pyrogallol autooxidation and is expressed as units/mg protein.

- *Reduced Glutathione (GSH):*

Estimated using Ellman's method (1959). Sample homogenate was treated with 20% TCA-EDTA, centrifuged, and the supernatant reacted with Ellman's reagent. Absorbance was measured at 412 nm and compared with a standard GSH curve.

- *Glutathione Peroxidase (GSHPx):*

Measured following Wood's method (1970). Cytosolic fractions were incubated with phosphate buffer, NaN_3, NADPH, EDTA, and glutathione reductase. The reaction was initiated with H_2O_2, and NADPH consumption was monitored at 340 nm. Activity was expressed as mg protein.

IV. RESULTS AND DISCUSSION

Antioxidant tests were performed *in-vivo* and *in-vitro*. Obtained results are presented following:

➢ *In Vitro Antioxidants Activities Test*

- *Reducing Power Assay:*

The reducing power assay (Table 1, Graph 1) revealed a concentration-dependent increase in antioxidant activity for all tested samples, with ascorbic acid (standard) demonstrating the highest percent inhibition across all concentrations. At 500 µg/mL, ascorbic acid showed 84.10% inhibition, significantly surpassing both *Hibiscus rosa-sinensis* (China rose) bark extract (41.99%) and *Trigonella foenum-graecum* (fenugreek) seed extract (31.25%). At the lowest concentration (25 µg/mL), inhibition was 43.55%, 17.72%, and 6.89% for ascorbic acid, China rose, and fenugreek respectively, indicating that while both extracts possess reducing power, they are considerably weaker than the standard. China rose consistently outperformed fenugreek at each concentration, suggesting a higher antioxidant potential. These findings align with previous studies highlighting phenolic compounds and flavonoids in both plants as key contributors to their antioxidant properties (Kumar *et al*., 2014; Kumari *et al*., 2020), although the differences in activity reflect variations in phytochemical composition and concentration.

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Table 1 % Inhibition in Reducing Power Assay of Standard and Extracts

Concentrations (µg/ml)	% Inhibition		
	Ascorbic acid	China rose	Fenugreek
25	43.55	17.72	6.89
50	47.13	29.01	11.63
100	55.39	33.87	16.93
250	71.78	37.25	27.56
500	84.1	41.99	31.25

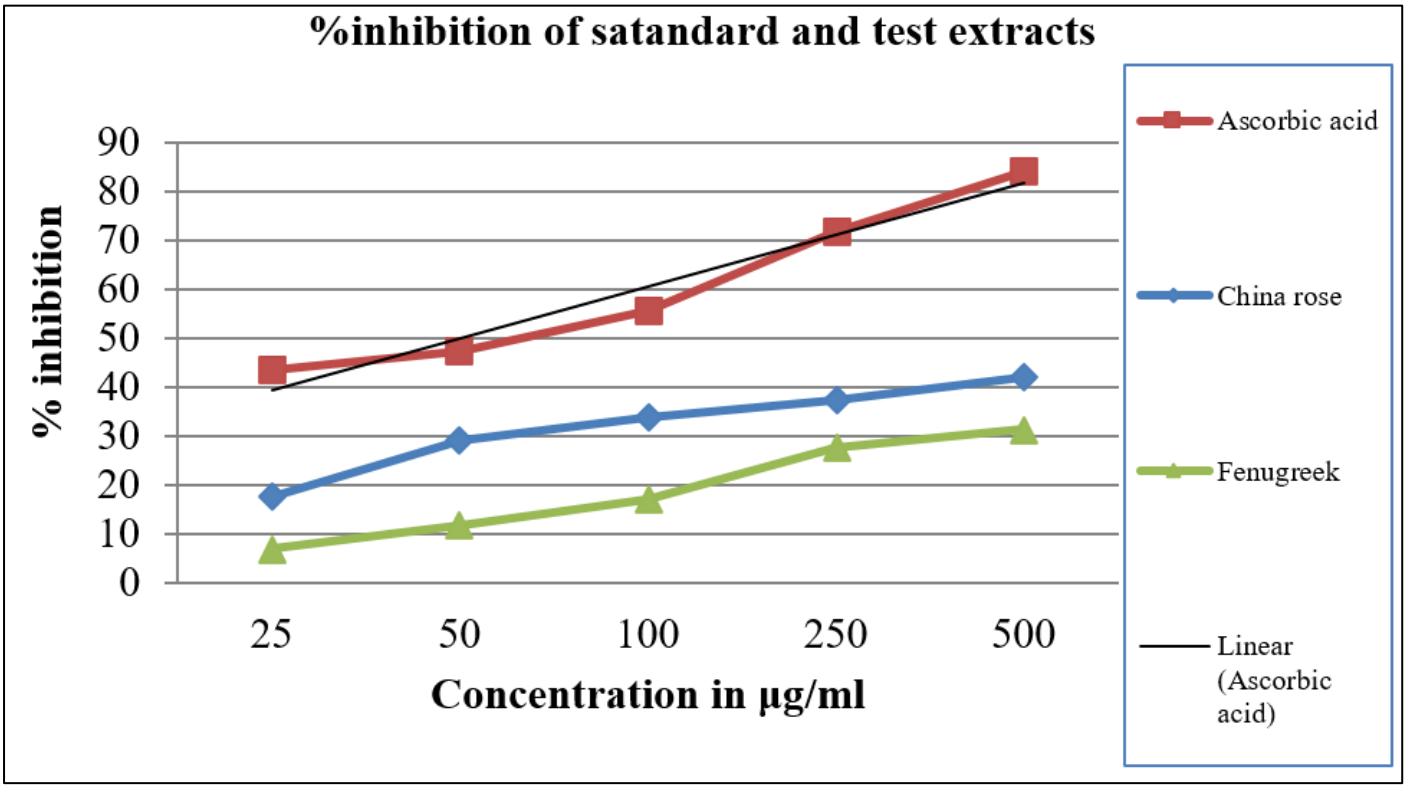

Fig 1 % Inhibition in Reducing Power Assay of Standard and Extracts

- *Hydrogen Peroxide Scavenging Activity:*

The hydrogen peroxide scavenging assay (Table 2, Graph 2) demonstrated that all tested samples exhibited concentration-dependent increases in antioxidant activity, with ascorbic acid (standard) showing the highest scavenging efficiency across all concentrations. At 250 µg/mL, ascorbic acid exhibited 82.97% inhibition of hydrogen peroxide, followed by fenugreek seed extract (59.86%) and *Hibiscus rosa-sinensis* (China rose) bark extract (48.36%). Interestingly, fenugreek consistently outperformed China rose at all concentrations, with notably higher inhibition at 25 µg/mL (38.20% vs. 30.15%) and at 100 µg/mL (55.21% vs. 36.77%). These results indicate that fenugreek extract has a stronger hydrogen peroxide neutralizing ability, possibly due to its rich content of phenolics, flavonoids, and saponins, which are known to scavenge reactive oxygen species (Kaviarasan *et al*., 2007; Kumari & Jain, 2020). Although both plant extracts demonstrated appreciable antioxidant potential, they remained less potent than the ascorbic acid control, highlighting their relative but significant free radical scavenging properties.

Table 2 % Inhibition in Hydrogen Peroxide Scavenging of Standard and Extracts

Concentrations (µg/ml)	% Inhibition		
	Ascorbic acid	China rose	Fenugreek
25	43.65	30.15	38.2
50	55.79	33.68	49.13
100	67.82	36.77	55.21
250	82.97	48.36	59.86
500	86.22	54.26	63.48

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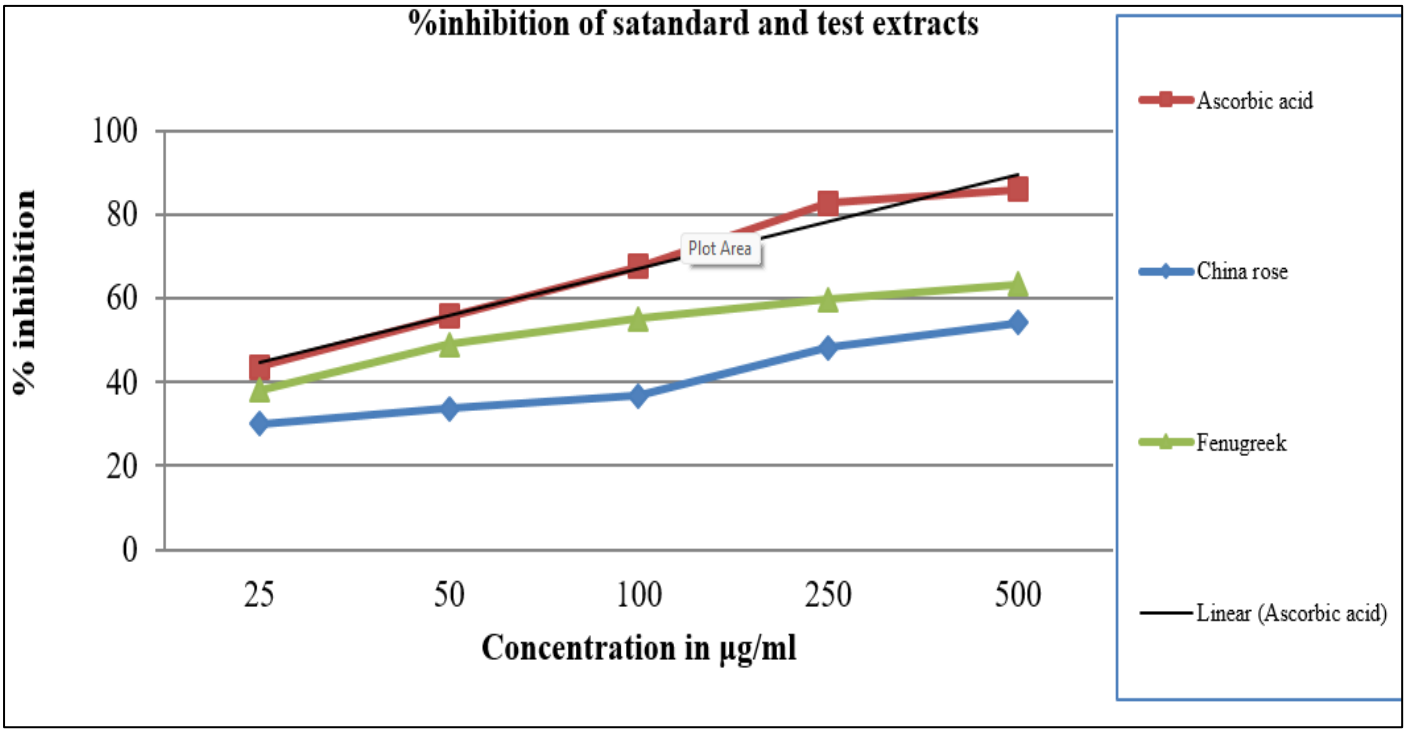

Fig 2 % Inhibition in Hydrogen Peroxide Scavenging of Standard and Extracts

- *DPPH (2,2-Diphenyl-1-Picrylhydrazyl) Free Radical Scavenging Assay:*

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay (Table 3, Graph 3) is a widely employed method to evaluate the free radical scavenging ability of antioxidant compounds. In this study, ascorbic acid exhibited the highest percent inhibition at all concentrations, reaching 85.34% at 500 µg/mL, confirming its strong radical scavenging capacity. Among the plant extracts, *Trigonella foenum-graecum* (fenugreek) seed extract showed consistently greater inhibition compared to *Hibiscus rosa-sinensis* (China rose) bark extract. At the highest tested concentration (500 µg/mL), fenugreek exhibited 65.24% inhibition, while China rose reached 52.41%. Even at the lowest concentration (25 µg/mL), fenugreek (32.62%) surpassed China rose (26.12%). This trend suggests a higher DPPH radical neutralizing potential in fenugreek, likely attributable to its richer content of polyphenols and flavonoids, which are known to donate hydrogen atoms or electrons to stabilize free radicals (Kaviarasan *et al*., 2007; Kumari & Jain, 2020). Although both extracts showed dose-dependent activity, their antioxidant strength remained lower than the standard, highlighting their potential but comparatively moderate efficacy in DPPH scavenging.

Table 3 % Inhibition in DPPH Test of Standard and Extracts

Concentrations (µg/ml)	% Inhibition		
	Ascorbic acid	China rose	Fenugreek
25	43.2	26.12	32.62
50	55.22	33.31	41.56
100	67.13	36.36	49.1
250	82.12	45.62	57.48
500	85.34	52.41	65.24

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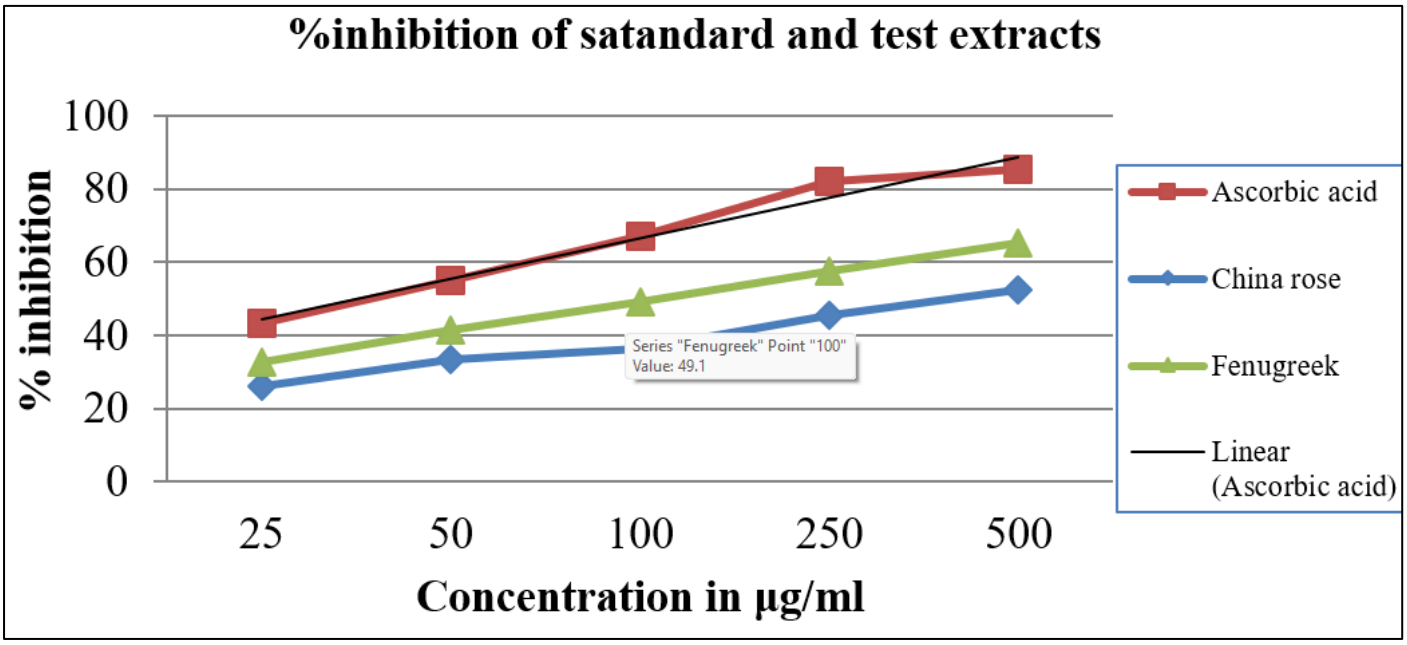

Fig 3 % Inhibition in DPPH Test of Standard and Extracts

➢ *Study of Control and Anaemia Induced Mice:*

Impact study of phenylhydrazine-induced anaemia on the antioxidant defense system in mice by comparing enzymatic antioxidant levels between control and anaemia-induced groups. Obtained data are presented in table 4 and graph 4.

Table 4 Antioxidant Levels between Control and Anaemia-Induced Groups.

Parameters	Control		Anaemia Induced
	1st Day	11th Day	11th Day
SOD (Units/mg protein)	5.40±0.80	5.41±0.85	0.80 ± 0.20
GSH (nm of GSH/mg protein)	4.10±0.72	4.11±0.76	1.10 ± 0.39
GSHPx (Unit/mg protein)	1.90±0.56	1.90±0.53	0.70 ± 0.21

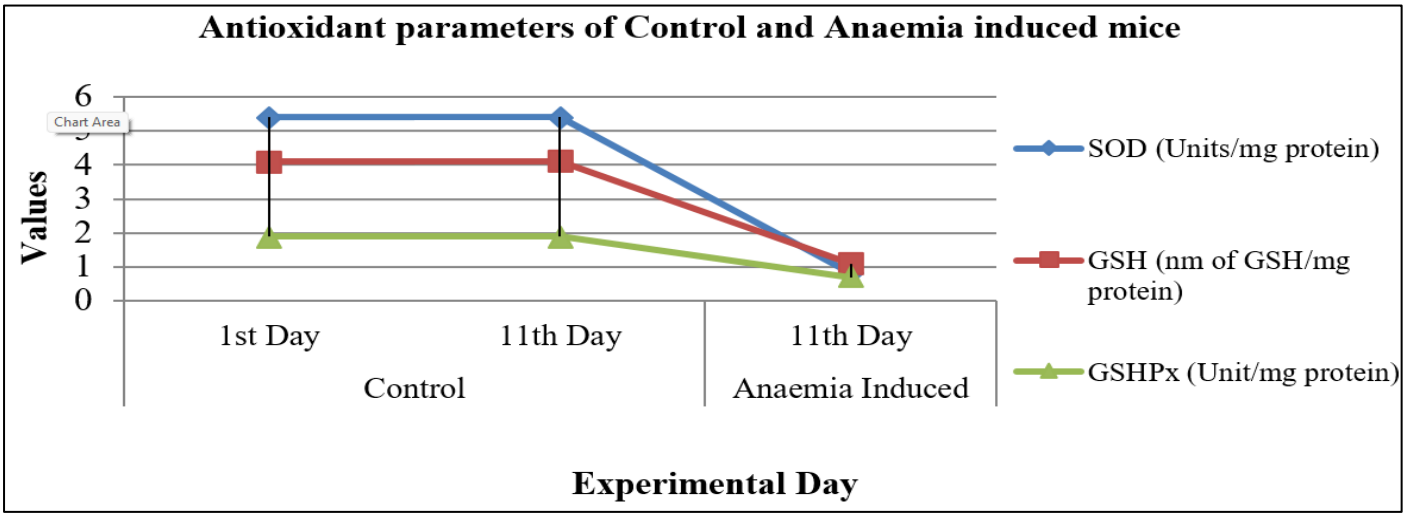

Fig 4 Antioxidant Levels between Control and Anaemia-Induced Groups.

- *Superoxide Dismutase (SOD):*

SOD is a critical enzyme in the cellular defense mechanism against oxidative stress, catalyzing the dismutation of superoxide radicals into oxygen and hydrogen peroxide. In the control group, SOD levels remained stable from Day 1 to Day 11 (~5.40 units/mg protein). However, in the anaemia-induced group, there was a drastic reduction to 0.80 ± 0.20 units/mg protein, indicating substantial oxidative damage. Phenylhydrazine (PHZ) is a known hemolytic agent that induces oxidative stress by generating reactive oxygen species (ROS), particularly superoxide anions, which overwhelm endogenous antioxidant defenses like SOD (Thomas *et al*., 2013). The significant decline in SOD activity confirms the ROS-mediated oxidative injury associated with PHZ-induced haemolysis (Ravi *et al*., 2004).

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- *Reduced Glutathione (GSH):*

GSH is a major non-enzymatic antioxidant that maintains cellular redox homeostasis and detoxifies free radicals and peroxides. The GSH levels in the anaemic group decreased significantly from the control value (4.11 ± 0.76 to 1.10 ± 0.39 nmol/mg protein), reflecting GSH depletion due to excessive ROS generation. Depletion of GSH is a hallmark of oxidative stress and cellular injury in anaemia models. Studies have shown that PHZ administration leads to rapid GSH oxidation and depletion in red blood cells, enhancing lipid peroxidation and contributing to membrane fragility and haemolysis (Jain *et al*., 1990; Sinha *et al*., 2013).

- *Glutathione Peroxidase (GSHPx):*

GSHPx reduces hydrogen peroxide and lipid hydroperoxides using GSH as a substrate, thereby protecting cells from oxidative damage. Control mice maintained GSHPx activity at 1.90 ± 0.53 units/mg protein, whereas anaemic mice showed a sharp decline to 0.70 ± 0.21 units/mg protein. This reduction in GSHPx activity is consistent with oxidative stress conditions, where the enzyme becomes overwhelmed or inactivated due to GSH depletion and high peroxide levels. Similar reductions in GSHPx have been reported in anemic and hemolytic models due to excessive ROS exposure (Ozen *et al*., 2004; Priscilla & Prince, 2009).

➢ *Study of Experimental groups:*

Impact study of phenylhydrazine-induced anaemia on the antioxidant defense system in mice by comparing enzymatic antioxidant levels between experimental groups is presented in table 5.

Table 5 Antioxidant Levels between Experimental Groups

Studied Day	Studied Groups	SOD (Units/mg protein)	GSH (nm of GSH/ mg of protein)	GSHPx (Unit/ mg of proteins)
	I(A)	5.41±0.85	4.11±0.76	1.90±0.53
	I(B)	0.80 ± 0.20	1.10 ± 0.39	0.70 ± 0.21
	I(C)	5.41±0.85	4.11±0.76	1.90±0.53
	I(F)	5.41±0.85	4.11±0.76	1.90±0.53
	I(G)	5.41±0.85	4.11±0.76	1.90±0.53
	II(A)	5.41±0.85	4.11±0.76	1.90±0.53
	II(D)	0.80 ± 0.20	1.10 ± 0.39	0.70 ± 0.21
	II(E)	0.80 ± 0.20	1.10 ± 0.39	0.70 ± 0.21
	II(H)	0.80 ± 0.20	1.10 ± 0.39	0.70 ± 0.21
	II(I)	0.80 ± 0.20	1.10 ± 0.39	0.70 ± 0.21
	II(J)	0.80 ± 0.20	1.10 ± 0.39	0.70 ± 0.21
	II(K)	0.80 ± 0.20	1.10 ± 0.39	0.70 ± 0.21
1st Day	II(S)	0.80 ± 0.20	1.10 ± 0.39	0.70 ± 0.21
	I(A)	5.42±0.91	4.11±0.82	1.91±0.57
	I(B)	0.77 ± 0.19	1.06 ± 0.38	0.67 ± 0.19
	I(C)	5.44±0.91	4.13±0.82	1.92±0.57
	I(F)	5.45±0.91	4.14±0.82	1.92±0.57
	I(G)	5.46 ± 0.91	4.15 ± 0.82	1.93 ± 0.57
	II(A)	5.48 ± 0.91	4.16 ± 0.82	1.93 ± 0.57
	II(D)	1.52 ± 0.38	2.09 ± 0.74	1.33 ± 0.40
	II(E)	1.64 ± 0.41	2.26 ± 0.80	1.44 ± 0.43
	II(H)	1.58 ± 0.40	2.18 ± 0.77	1.39 ± 0.42
	II(I)	1.73 ± 0.43	2.38 ± 0.84	1.51 ± 0.45
	II(J)	1.62 ± 0.41	2.23 ± 0.79	1.42 ± 0.43
	II(K)	1.80 ± 0.45	2.48 ± 0.88	1.58 ± 0.47
15th Day	II(S)	2.02 ± 0.50	2.77 ± 0.98	1.76 ± 0.53
	I(A)	5.44±0.89	4.13±0.77	1.91±0.63
	I(B)	0.76 ± 0.19	1.05 ± 0.38	0.67 ± 0.19
	I(C)	5.46±0.89	4.15±0.77	1.92±0.63
	I(F)	5.47±0.89	4.15±0.77	1.93±0.63
	I(G)	5.48 ± 0.89	4.17 ± 0.77	1.93 ± 0.63
	II(A)	5.50 ± 0.89	4.18 ± 0.77	1.93 ± 0.63
	II(D)	1.68 ± 0.42	2.31 ± 0.82	1.47 ± 0.44
	II(E)	1.74 ± 0.44	2.40 ± 0.85	1.53 ± 0.46
	II(H)	1.73 ± 0.43	2.38 ± 0.84	1.51 ± 0.45
	II(I)	1.82 ± 0.45	2.50 ± 0.89	1.59 ± 0.48
	II(J)	1.77 ± 0.44	2.43 ± 0.86	1.55 ± 0.46
	II(K)	1.87 ± 0.47	2.57 ± 0.91	1.64 ± 0.49
30th Day	II(S)	2.27 ± 0.57	3.12 ± 1.11	1.99 ± 0.60
	I(A)	5.43±0.81	4.12±0.72	1.91±0.53
	I(B)	0.75 ± 0.19	1.03 ± 0.38	0.66 ± 0.19
	I(C)	5.45±0.81	4.14±0.72	1.92±0.53
	I(F)	5.46±0.81	4.14±0.72	1.92±0.53
45th Day	I(G)	5.47 ± 0.81	4.16 ± 0.72	1.93 ± 0.53

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	II(A)	5.49 ± 0.81	4.17 ± 0.72	1.93 ± 0.53
	II(D)	1.76 ± 0.44	2.42 ± 0.86	1.54 ± 0.46
	II(E)	1.81 ± 0.45	2.49 ± 0.88	1.58 ± 0.47
	II(H)	1.82 ± 0.45	2.50 ± 0.89	1.59 ± 0.48
	II(I)	1.86 ± 0.47	2.56 ± 0.91	1.63 ± 0.49
	II(J)	1.86 ± 0.47	2.56 ± 0.91	1.63 ± 0.49
	II(K)	1.93 ± 0.48	2.65 ± 0.94	1.69 ± 0.51
	II(S)	2.34 ± 0.59	3.22 ± 1.14	2.05 ± 0.62
	I(A)	5.42±0.83	4.11±0.77	1.91±0.56
	I(B)	0.74 ± 0.19	1.02 ± 0.37	0.65 ± 0.19
	I(C)	5.44±0.83	4.13±0.77	1.92±0.56
	I(F)	5.45±0.83	4.14±0.77	1.92±0.56
	I(G)	5.46 ± 0.83	4.15 ± 0.77	1.93 ± 0.56
	II(A)	5.48 ± 0.83	4.16 ± 0.77	1.93 ± 0.56
	II(D)	1.92 ± 0.48	2.64 ± 0.94	1.68 ± 0.50
	II(E)	1.96 ± 0.49	2.70 ± 0.96	1.72 ± 0.51
	II(H)	1.97 ± 0.49	2.71 ± 0.96	1.72 ± 0.52
	II(I)	2.02 ± 0.50	2.77 ± 0.98	1.76 ± 0.53
	II(J)	2.01 ± 0.50	2.76 ± 0.98	1.76 ± 0.53
	II(K)	2.09 ± 0.52	2.87 ± 1.02	1.83 ± 0.55
60th Day	II(S)	2.50 ± 0.62	3.43 ± 1.22	2.18 ± 0.65

- *Superoxide Dismutase (SOD):*

SOD is a frontline defense against reactive oxygen species (ROS), catalyzing the dismutation of superoxide radicals into hydrogen peroxide and molecular oxygen (McCord & Fridovich, 1969). A marked depletion in SOD activity was observed (Graph 5.) in anaemia-induced mice across all time points, dropping to 0.74 ± 0.19 units/mg protein by Day 60 compared to 5.42 ± 0.83 units/mg protein in the control group. This decrease is consistent with the oxidative damage caused by PHZ, which generates superoxide radicals and disrupts erythrocyte integrity (Thomas *et al.*, 2013). Treatment with China rose and fenugreek extracts significantly improved SOD activity, particularly at higher doses and in combination regimens, suggesting potent antioxidant efficacy. By Day 60, the combination group (400 mg/kg) reached 2.09 ± 0.52 units/mg, approaching the effect of ferrous sulfate (2.50 ± 0.62).

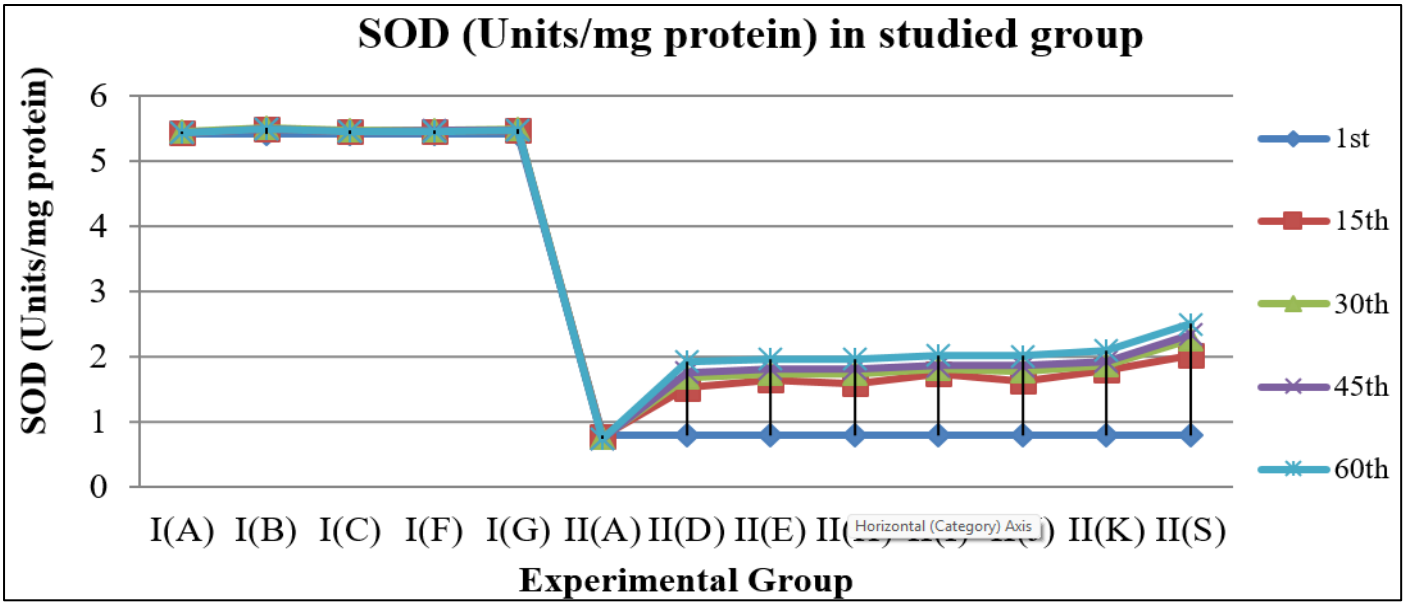

Fig 5 SOD levels Between Experimental groups

- *Reduced Glutathione (GSH):*

GSH, a major intracellular antioxidant, plays a central role in maintaining redox balance and neutralizing peroxides (Jain *et al.*, 1990). Anaemia induced by PHZ significantly reduced GSH levels (Graph 6.), indicating excessive oxidative load and compromised detoxification. However, groups treated with China rose, fenugreek, and their combinations showed progressive restoration of GSH, reaching up to 2.87 ± 1.02 nmol/mg protein by Day 60 in the combination group (400 mg/kg), again closely matching the ferrous sulfate-treated group (3.43 ± 1.22). These findings align with reports showing phytochemical antioxidants enhancing GSH biosynthesis and recycling (Sinha *et al.*, 2013).]

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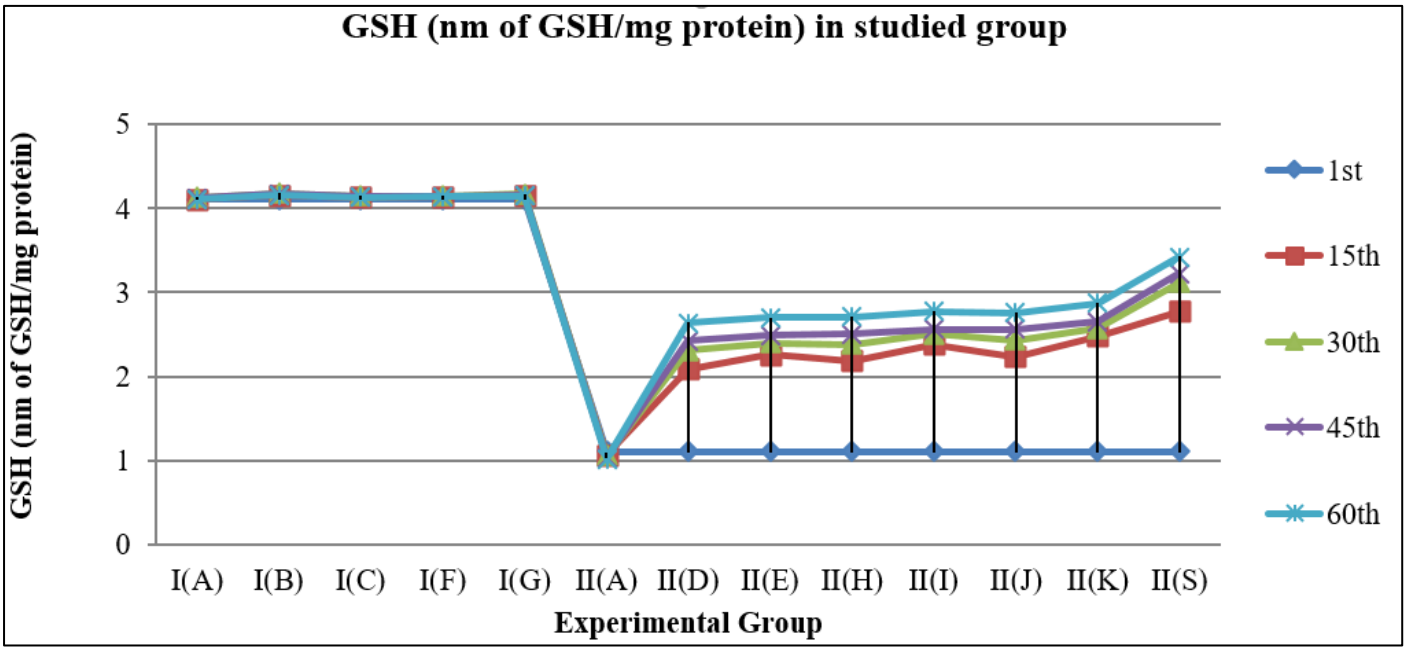

Fig 6 GSH levels between Experimental Groups.

➢ *Glutathione Peroxidase (GSHPx):*

GSHPx catalyzes the reduction of hydrogen peroxide and lipid peroxides, thereby preventing oxidative cell damage (Flohé & Günzler, 1984). GSHPx levels (Graph 7.) declined significantly in anaemic animals, confirming oxidative stress and GSH exhaustion. A gradual and sustained improvement was observed in treated groups, with the highest increase in the combination treatment (1.83 ± 0.55) and ferrous sulfate (2.18 ± 0.65) by Day 60. These results underscore the role of China rose and fenugreek in supporting enzymatic antioxidant defenses during haemolytic stress.

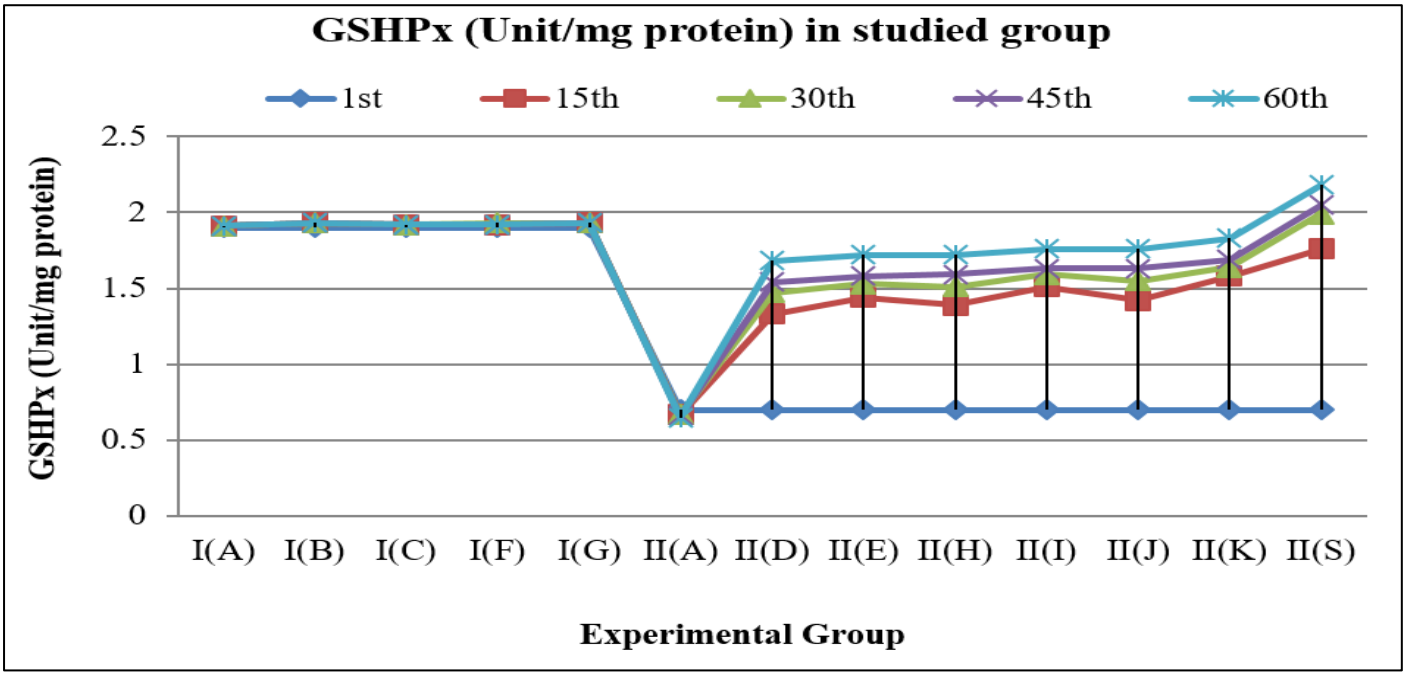

Fig 7 GSHPx Levels between Experimental Groups.

V. CONCLUSION

The study demonstrates that both *Trigonella foenum-graecum* seed and *Hibiscus rosa-sinensis* bark extracts possess significant antioxidant activities in phenylhydrazine-induced anaemic mice. Their ability to restore antioxidant enzyme levels suggests their potential role in managing oxidative stress-associated anaemia. Furthermore, the absence of acute or sub-acute toxicity at therapeutic doses highlights their safety for further pharmacological development. These results validate the traditional use of these plants and pave the way for future studies aimed at clinical application.

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