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Anti-Anemic and Haemopoietic Evaluation of *Trigonella foenum-graecum* (Fenugreek) in Rodent Model

Anand Chourasiya*[1], Rakesh Kumar Sahu[1], Mohd Azaz Khan[2]

[1] Millennium College of Pharmacy, Nathu Barkhera Road, Neelbad, Madhya Pradesh 462044

[2] Pinnacle Biomedical Research Institute (PBRI), Near, Bharat Scout and Guides Campus, Shanti Marg, Shyamla Hills Road, Bhopal, Madhya Pradesh 462003

ABSTRACT

The health benefits and medicinal properties of herbal food products are known since antiquity. Fenugreek, *Trigonella foenum-graecum* Linn (*T. foenum-graecum*, Fabaceae), a seed spice used to enhance flavor, color and texture of food is employed for medicinal purposes in many traditional systems. Ethno botanical survey of *T. foenum-graecum* revealed the seeds of the plant to be useful in anemia. The objective of this study was to study the ant-anemic effect of hydroalcoholic extract of seeds of *T. foenum-graecum* against phenylhydrazine induced anemic rat model. The hydroalcoholic extracts of seeds were prepared by soxhlation. Phytochemical analysis of the extracts was performed using standard testing procedures. Hemolytic anemia was induced in male Wistar rats by intraperitoneal administration of phenylhydrazine HCl (PHZ) at doses of 40 mg/kg of body weight during two successive days then one day after the animals were treated orally by the hydroalcoholic extracts with the amounts of 200 mg/kg and 400 mg/kg of body weight and Dexorange (reference drug) up to 13 days. The rats were analyzed for hematological parameters such as hemoglobin (Hb), red blood cell count (RBC) and white blood cell count (WBC) on day 2 and 13. Phytochemical screening of the extracts indicated the presence of carbohydrates, saponins, sterols, polyphenols, tannins and flavonoids. Anemia was induced successfully in Groups II, III, IV and V which was indicated by a mean reduction of 51.6% in RBC count; 52.85% in Hb content and 54.9% in WBC. Analysis of hematological parameters on day 13 showed that extract significantly ($p<0.05$) improved Hb, RBC and WBC count at a dose of 400 mg/kg body weight. This study, not only substantiates the folklore use of the seed of *T. foenum-graecum,* but also suggests its inclusion in the treatment of anemia as it exhibited significant anti-anemic activity

Keywords: *Trigonella foenum-graecum,* Anemia, Phytochemical analysis, Phenyl hydrazine, Dexorange

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***Address for Correspondence:**

Anand Chourasiya, Millennium College of Pharmacy, Nathu Barkhera Road, Neelbad, Madhya Pradesh 462044

INTRODUCTION

Certain diseases such as malaria, malnutrition, protozoa infections and pregnancy are among various conditions that may lead to anemia in both adults and children. Anemia is a condition, wherein the quantity of circulating hemoglobin (Hb) in the blood is <13 g/dl for male and <12 g/dl for female adult[1]. There are several types of anemia and all are characterized by a reduction in a number of circulating red blood cells (RBC) and Hb[2]. The consequences of anemia included general body weakness, frequent tiredness and lowered resistance to disease. Anemic condition if not treated, may lead to serious problems in pregnant women such as premature delivery and low birth weight. It is of concern in children in whom anemia is associated with impaired mental and physical development. Anemia is a condition commonly seen in developing countries because of lack of nutrition and frequent use of drugs to treat diseases. Hemolytic anemia is a form of inherited or acquired anemia resulting from either intravascular or extravascular RBC destruction. It has numerous external and internal causes which are either relatively harmless or are life-threatening in nature[3]. The exposure too many chemicals have also been associated with RBC destruction and hemolytic anemia[4]. The hemolytic activity of aryl hydrazines, such as phenyl hydrazine, dapsone, hydroxylamine, divicine, may lead to acute hemolytic anemia in vertebrates[5]. Yeshoda induced anemia in rats following a single intraperitoneal administration of phenylhydrazine hydrochloride (PHZ) at a dose of 20 mg/kg b.w. (aqueous solution). Erythrocyte concentration lowered to about 50% and Hb level to about 60% of normal values in the course of 4 days[6]. Plant and plant products are being utilized as a source of medicine since long. Plant extracts are used as phytotherapeutics and

are still a large source of natural antioxidants. Natural antioxidants strengthen the endogenous antioxidant defense from ROS ravage and restored the optimal balance by neutralizing the reactive species[7]. Particularly, flavonoids and phenolics are considered as potential therapeutic agents. A wide range of ailments and is widely distributed in the plant kingdom and, therefore, an integral part of the diet with the significant amount reported in vegetables, fruits and beverages[8]. From ancient time, medicinal plants classified as Rasayana in Ayurveda are believed to be useful in strengthening the hematopoietic and immune system of an individual. Ayurvedic physicians suggested various herbs for the treatment of hematological disorders as a source of iron and other minerals. Silja et al. (2008) included *Ageratum conyzoides, Boerhavia diffusa, Centella asiatica, Hemidesmus indicus, Ichnocarpus frutescens, Momordica charantia, Moringa oleifera, Phyllunthus amarus, Phyllunthus emblica, Punica granatum, Ocimum tenuiflorum, Solanum americanum* as useful plants in the treatment of anemia[9]. *Adenia gummifera, Allophylus rubifolius, Albizia versicolor, Brackenridgea zanguebarica, Bridelia cathartica, Comniphora africana, Hibiscus sabdariffa*, *Lannea stuhlmanni, Sorgum bicolor, Theobroma cacao, Triumfetta rhomboidea* was also reported to be used in conditions of anemia[10-12].

Various researchers successfully evaluated the potential of several medicinal plants in the treatment of anemia using various experimental animal models. The hematinic activity of an orally administered aqueous extract of *Hibiscus cannabinus* leaves was evaluated in phenylhydrazine (10 mg/kg, p.o, for 8 days) induced anemic rats[13]. *Tectona grandis* leaves were evaluated on anemia model of rat induced by intraperitoneal injection of phenyl hydrazine at 40 mg/kg for 2 days[14].

Fenugreek is an annual plant of the family Fabaceae. Fenugreek seeds have been used for a long time as a spice and to treat several diseases. Charred fenugreek seeds have been recovered from Iraq (radiocarbon dating to 4000 BC) and desiccated seeds have also found in the tomb of Tutankhamen[15]. There is mention of fenugreek seed as a natural healer in different folk and tribal literatures. In Ayurveda, fenugreek seed extract has been mentioned as an effective blood tonic, which helps in curing anemia during menstruation in women. Ibrahium and Hegazi (2009) showed that the biscuits made from fenugreek seed flour increased iron bioavailability, blood hemoglobin, hematocrit and serum iron content in experimental animal model[16]. Mahmud et al. (2012) showed that fenugreek leaf extract and seed extract caused significant increase in hematological and biochemical parameters including blood hemoglobin, hematocrit, total count of RBC, serum total iron binding capacity, serum protein and minerals (iron and zinc) in animal model when compared to anemia control group[17]. A clinical study in females of child bearing age indicated that fenugreek seed extract has good beneficial effect to raise blood hemoglobin[18]. Fenugreek seed extract has already shown to have antioxidant activities. Naidu et al. (2011) showed that the extracts of fenugreek husk, seed and endosperm exhibited 72%, 64% and 56% antioxidant activity respectively by free radical scavenging method[19]. They also mentioned the presence of saponin, protein, polyphenols etc. in fenugreek seed to exhibit antioxidant activity. Previous works ensured the role of antioxidant in the pathogenesis of iron deficiency anemia[20]. WHO declared iron deficiency anemia as one of the most important contributing factors to the global burden of diseases[21]. Present remedies available to treat iron deficiency may cause severe adverse effects including nausea, diarrhea, vomiting, anaphylaxis etc. The objective of this study aims at investigating the therapeutic benefit of the plant in the treatment of anemia.

MATERIAL AND METHODS

Plant material

The seeds of plant *T. foenum-graecum* were purchased from local market of Bhopal (M.P.).The sample was identified by Botanist, Department of Botany, Safia College of Arts and Science, peer gate Bhopal. A herbarium of plants was submitted to the specimen library of Safia College of Arts and Science, peer gate Bhopal and The specimen voucher no. of *T. foenum-graecum* L is 154/Bot/Saf/Science/College.

Chemical reagents

All the chemicals used in this study were obtained from Hi Media Laboratories Pvt. Ltd. (Mumbai, India), Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India).All the chemicals used in this study were of analytical grade.

Extraction

Seeds of *T. foenum-graecum* were purchased, washed and rinsed properly. They were dried in shade. Initially 250 gm of crude powder will be taken and packed in a packing paper. This pack will be placed in a soxhlet extractor & extracted with hydroalcoholic solvent (70:30); the extraction will be carried out until the drug was completely extracted. The extract will be then filtered with whatman filter papers (No.1) and the filtrate will be evaporated to dryness in rotary evaporator at 40ºC. The obtained crude extract will be stored in an air tight container free from any contamination until it was used. Finally the percentage yields were calculated of the dried extracts[22].

Animals

Albino rats of either sex (250±25g body weight) were used for this study. They were housed in groups of three in polypropylene cage at ambient temperature (25±2°C), relative humidity (55±5%) and 12 hrs/12 hrs light-dark cycles. Animals had free access to commercial brand rat pellet diet and water given *ad libitum*. The protocol of the experiment was approved by the Institutional Animal Ethical Committee (IAEC) of Pinnacle Biomedical Research Institute (PBRI) Bhopal (Reg No. 1824/PO/ERe/S/15/CPCSEA) as per the guidance of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Protocol Approval Reference No. PBRI/IAEC/PN-18017. The studies were conducted according to the guidelines of CPCSEA. The standard gastric cannulas will be used for oral drug administration in experimental animals.

Acute oral toxicity

Acute toxicity study of the prepared seeds extracts was carried out according to the Organization for Economic Co-Operation and Development (OECD) Guidelines-423. The animals were fasted for 4 h, but allowed free access to water throughout. As per the OECD recommendations, the starting dose level should be that which is most likely to produce mortality in some of the dosed animals; and when there is no information available on a substance to be tested in this regard; for animal welfare reasons, three animals of single sex are used for each step. The dose level to be used as the starting dose is selected from one of three fixed levels 5, 300 and 2000 mg/kg body weight. Acute toxicity was determined as per reported method[23].

Animal's treatment

Anemia induction

Anemia was induced by intraperitoneal phenylhydrazine injection (40 mg/kg/body weight) during two days (Day 0 and Day 1) [24,25].

Experimental design

The animals were distributed according to weight in five groups each of six rats and received by gavage during 13days *T. foenum-graecum* extracts and Dexorange (reference drug) as follows:

Group I (Normal): Normal control received saline.

Group II (PHZ control): Anaemic control received phenylhydrazine (40 mg/kg) at day 0 and day 1 then distilled water daily during 13 days.

Group III (TFG 200): In this group rats were treated with phenylhydrazine (40 mg/kg) at day 0 (D0) and day 1 (D1) then treated with *T. foenum-graecum* extract (200 mg/kg) daily during 13 days.

Group IV (TFG 400): In this group rats were treated with phenylhydrazine (40 mg/kg) at day 0 (D0) and day 1 (D1) then treated with *T. foenum-graecum* extract (400 mg/kg) daily during 13 days.

Group V (Dexorange): Standard control received phenylhydrazine (40 mg/kg) at days 0 (D0) and 1 (D1) and dexorange single dose 200 mg/kg per day for 13 day.

The extracts and dexorange treatment began one day after phenylhydrazine administration. On completion of the experimental period, animals were anesthetized with thiopentone sodium (50 mg/kg, ip). The blood was collected with and without EDTA as an anticoagulant. Plasma was separated by centrifugation. Plasma was used for the estimation of various biochemical parameters. Hemoglobin was estimated by cyanomethaemoglobin method[26]. WBC and RBC were counted by Ochei method, 2000[27].

Statistical analysis

The values expressed as Mean ± standard deviation (SD) from 6 animals. In each group, the various means were compared with those of day 0 (D0) then day 2 (D2) by using one way analysis of variance (ANOVA) followed by Dunnett's test. The statistical difference was considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Results

The crude extracts so obtained after the soxhlation extraction process were concentrated on water bath by evaporation the solvents completely to obtain the actual yield of extraction. The yield of extracts obtained from the seeds of the plants using hydroalcohol as solvents was found to be 9.43 %. The results of qualitative phytochemical analysis of the crude powder seeds of *T. foenum-graecum* are shown in Table 1. Hydroalcoholic extracts of seeds sample of *T. foenum-graecum* showed the presence of flavonoids, phenols, tannins, carbohydrate, glycosides and proteins. The present study was carried out to evaluate anti anemic properties of hydroalcoholic extracts of *T. foenum-graecum* seeds. Hematological parameters such as red blood cells number (RBC), hemoglobin concentration (Hb) and white blood cells number (WBC) were given before treatment (D0), after phenylhydrazine induced anemia (D2), at first, second and 13 days of treatment. The mean values for whole animals groups before phenylhydrazine injection were 7.84±0.252x 10^6/µL, 14.51±0.902 g/dL and 8.073±0.338 10^3/µL respectively for RBC, Hb, and WBC. The intraperitoneal phenylhydrazine injection during two successive days involved a significant decrease ($P < 0.001$) red blood cells (3.83±0.329 x10^6/µL), hemoglobin concentration (8.11±0.540 g/dL) and white blood cells (4.16±0.823 x10^3/µL) in anemia groups compared to day 0 (D0). During treatment, results showed a strong increase in RBC at 13 days in groups treated with Dexorange (8.28±0.459), hydroalcoholic extracts 200 mg/kg (7.15±0.944) and 400 mg/kg (8.31±0.554) compared to day 2. But red blood cells increase in untreated rats was progressive and reached 8.21±0.410 (Table 2 & Figure 1). Hemoglobin concentration was strongly restored ($P < 0.001$) in groups treated with Dexorange, hydroalcoholic extracts 200 mg/kg and 400 mg/kg of body weight respectively to reach 14.54±0.411, 12.44±0.551 and 14.04±0.355 compared to phenylhydrazine toxic effect at day 2. The percentage variation obtained at 13 days in untreated group was statistically identical ($P > 0.05$) to those obtained at second day in treated groups with dexorange and *T. foenum-graecum* extracts (Table 3& Figure 2). White blood cells: The administration of phenylhydrazine also decreased WBCs at day D2. This decrease was (4.165± 0.823) at 2nd day and (5.876±0.674) at 13th day in untreated rats G2, the rats of groups G3, G4, and G5 the increased of WBCs at day 13 was (8.2±0.548), (8.43±0.447), (8.63±0.572) respectively (Table 4& Figure 3).

Table 1 Phytochemical evaluation of *T. foenum-graecum* seeds extracts

S. No.	Experiment	Hydroalcoholic extract	
		Present	Absent
1	**Alkaloids**		
1.1	Mayer's reagent test	✓	-
1.2	Wagner's reagent test	✓	-
1.3	Hager's reagent test	✓	-
2.	**Carbohydrates**		
2.1	Molish's test	✓	-
2.2	Fehling's test	✓	-
2.3	Benedict's test	✓	-
2.4	Barfoed's test	✓	-
3	**Proteins and Amino Acids**		
3.1	Biuret test	✓	-
4.	**Flavonoids**		
4.1	Alkaline reagent test	✓	-
4.2	Lead Acetate test	✓	-
5.	**Glycoside**		
5.1	Borntrager test	-	✓
5.2	Legal's test	-	✓
5.3	Killer-Killiani test	-	✓
6.	**Tannin and Phenolic Compounds**		
6.1	Ferric Chloride test	✓	-
6.2	Lead Acetate test	✓	-
6.3	Gelatin test	✓	-
7.	**Saponin**		
7.1	Foam test	✓	-
8.	**Test for Triterpenoids and Steroids**		
8.1	Salkowski's test	✓	-
8.2	Libbermann-Burchard's test	✓	-

Table 2 Effect of administration of *T. foenum-graecum* extract on RBCs of iron deficient rats

S. No.	Treatment Groups	Day 0 (10^6/µl)	Day 2 (10^6/µl)	Day 13 (10^6/µl)
1	**Normal control**	8.17±0.302	7.16±0.046	8.21±0.410
2	**Positive control (PHZ 40 mg/kg)**	8.11±0.501	3.83±0.329	4.37±0.226
3	**TFG treated group 200mg/kg**	8.05±0.581	4.12±0.489	7.15±0.944
4	**TFG treated group 400mg/kg**	8.11±0.291	4.00±0.431	8.31±0.554
5	**Standard (200 mg/kg)**	8.12±0.325	3.72±0.537	8.28±0.459

Table 3 Effect of administration of *T. foenum-graecum* extract on hemoglobin of iron deficient rats

S. No.	Treatment Groups	Day 0 (gm/dL)	Day 2 (gm/dL)	Day 13 (gm/dL)
1	**Normal control**	14.75±0.377	14.3±0.201	14.5±0.071
2	**Positive control (PHZ 40 mg/kg)**	14.32±0.528	8.11±0.540	12.2±1.639
3	**TFG treated group 200mg/kg**	14.70±0.381	8.12±0.155	12.44±0.551
4	**TFG treated group 400mg/kg**	14.31±0.308	8.25±0.247	14.04±0.355
5	**Standard (200 mg/kg)**	14.55±0.322	8.19±0.684	14.54±0.411

Table 4 Effect of administration of *T. foenum-graecum* extract on WBCs of iron deficient rats

S. No.	Treatment Groups	Day 0 (10^3/µl)	Day 2 (10^3/µl)	Day 13 (10^3/µl)
1	**Normal control**	8.13±0.186	7.55±0.533	8.54±0.297
2	**Positive control (PHZ 40 mg/kg)**	7.37±0.705	4.165±0.823	5.876±0.674
3	**TFG treated group 200mg/kg**	8.08±0.622	5.3±0.648	8.2±0.548
4	**TFG treated group 400mg/kg**	8.05±0.368	5.00±0.700	8.43±0.447
5	**Standard (200 mg/kg)**	8.01±0.289	3.72±0.537	8.63±0.572

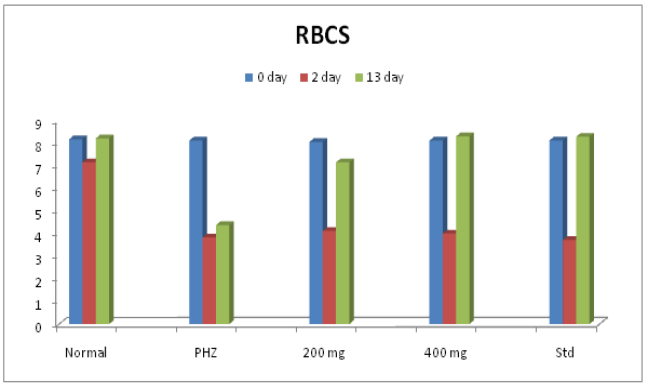

Figure 1 Effect of *T. foenum-graecum* extract on RBCs

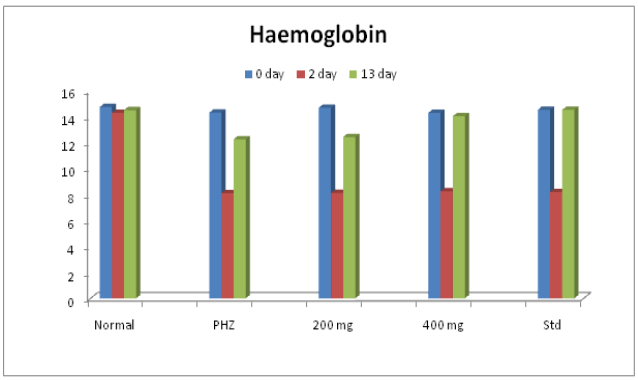

Figure 2 Effect of *T. foenum-graecum* extract on Hemoglobin

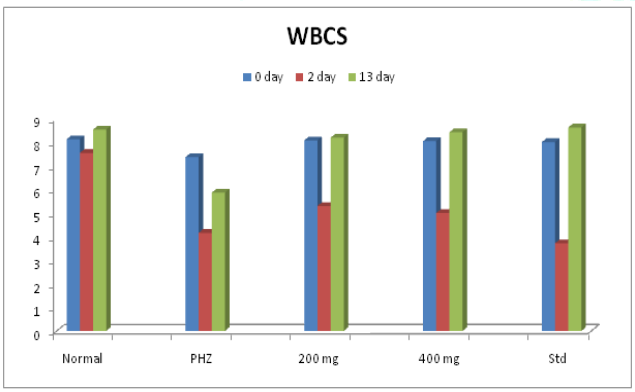

Figure 3 Effect of *T. foenum-graecum* extract on WBCs

DISCUSSIONS

Anaemia is one of the public health problems most widespread, especially in developing countries. It is characterized by the deficiency of red blood cells (RBC) or hemoglobin in the blood, which results in the disturbance of the oxygen transport. The normal rate of hemoglobin varies with age and gender. There is anaemia when the rate is less than 110 g / L for pregnant women and children of 6 months to 5 years, 120g / L for unpregnant women and 130 g/L for men[28]. There are two groups of anaemia, the lack of production of red blood cells (iron deficiency, aplastic or megaloblastic anaemia) or the abnormal destruction of red blood cells (hemolytic anaemia, or anaemia caused by a chronic disease). Iron deficiency anaemia is the most common type of anaemia. It is most widespread to children and women of all ages. The World Health Organization estimates that for the entire world, anaemia reached a staggering 2 billion people affected, also about 50% of cases is due to iron deficiency[28]. In the case of hemolytic anemia, the rate of production of red blood cells is normal or high, but they are destroyed too rapidly. This disease is acquired or inherited. Acquired, it may be due to a reaction of the immune system (autoimmune or allergic), in the presence of toxic substances in the blood (phenylhydrazine) or to the infections. Phenylhydrazine is a non-immunogenic drug that induces changes in the RBC membrane which leads to oxidative denaturation of haemoglobin. Formation of altered haemoglobin takes place known as “Heinz bodies” which decreases the life span of the erythrocytes[29]. This is characterized by increase in incidence of micronucleated polychromated and hypochromic erythrocytes resulting in increased mean cell volume and decreased mean cell Hb concentration levels[30].The prevalence of iron deficient anemia can be reduced by dietary supplementation with pharmaceutical preparations, iron fortification, and dietary diversification. Iron supplements are useful for production of a rapid improvement in the iron status in anemic individuals. Electrolytic iron and ferrous sulfate are widely used, but effectiveness depends on dietary bioavailability. The bioavailability of non-heme iron is between 2% and 20% and is influenced by a variety of inhibiting components in the diet[31]. Due to poor bioavailability, and other reasons, inorganic iron supplementation has limitations. Heme iron is absorbed via a heme transporter that is predominantly expressed in brush border membranes of duodenal enterocytes and hepatocytes[32]. In contrast, nonheme iron is transported into the body via nonselective iron channels[33] that are easily influenced by other metal ions. Therefore, absorption of heme iron in humans is more complete than absorption of non-heme iron. Due to good bioavailability, heme iron is recommended as an iron supplement. Phytochemical analysis revealed the presence of large chemical groups that are: alkaloids, flavonoids, polyphenols, sterols, terpenes, glycosides and saponins. They have antioxidant power, promote regeneration of tissue, reduce the permeability of blood capillaries and increase their resistance to hemolysis. The presence of these chemicals by their properties justifies the resistance of red blood cells of treated rats with the extract. The anti-anemic activity of *T. foenum-graecum* extract was assessed by determining the red blood cell count, hemoglobin and WBCs level. Phenylhydrazine decreased the RBC, Hb and WBCs as compared normal control.

CONCLUSION

The collective data of this study revealed that seeds have considerable anti-anemic activity as shown in PHZ-induced anemia in experimental rat model indicating the use of this plant for the treatment of anemia. Further studies are required to precisely define the bioactive and to develop suitable formulations to ensure maximum bioavailability and therapeutic efficacy.

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