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ANTI-ANEMIC ACTIVITY *Murraya Koenigii* LEAVES ON PHENYLHYDRAZINE INDUCED ANEMIA IN RATS

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ABSTRACT

The incidence of anemia is higher in the third world than in developed countries due to the presence of many aggravating factors such as poor nutrition, high prevalence of blood parasites. Anemia is one of the numerous ailments claimed to have been successfully treated with plant materials by traditional medicine practitioners. Many plant and plant products are used to treat the anemia. Keeping in view in the present study was evaluated the anti-anemic activity of *Murraya Koenigii* leaves. Phenylhydrazine, a alkylhydrazine was chosen to induce haemolytic anemia. The results of the present study concluded that *Murraya Koenigii* leaves inhibits anemia induced by phenylhydrazine. The anti-anaemic potentials of *Murraya Koenigii* leaves are lending credence to use of this plant in folklore medicine for the management of anemia.

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INTRODUCTION

Anemia is the most common blood disorder in developing countries especially in India that affects people of all ages, although the people at greater risk are the elderly, young women of child-bearing age and the infants. The incidence of a nemia is higher in the third world than in developed countries due to the presence of many aggravating factors such as poor nutrition, low socio-economic status, high prevalence of blood parasites such as *Plasmodium* and *Trypanosomes* and helminthic infections. Presently, more than half of the world's population would experience some form of anemia in their lifetime (Duff, 2008).

Among worldwide distribution of anemia approximately half of them are due to Iron deficiency anemia. Women are affected more than men and its widely prevalence is about 700- 800 million people in under developing countries and 60 – 70 million in developed countries. On a regional basis, South Asia and Africa have the highest prevalence with groups except rate of more than 40% in all age groups except for adult males and pregnant women, the latter group is the most vulnerable to anemia with an estimated prevalence rate of more than 65% in south Asia (Holden and Acomb, 2007). Hence, anemia is one of the leading health disorders posing a great threat to global healthcare.

Anemia is a medical condition in which the red blood cell count or hemoglobin is less than normal leading to reduced oxygen carrying capacity. The normal level of hemoglobin is generally different in males and females. For men, anemia is typically defined as hemoglobin level of less than 13.5 gram/dL and in women as hemoglobin of less than 12.0 gram/dL3. Infant has higher concentration 15g/dL at the time of birth and 9.5 g/dl at the time of 3 month. Normal life span of a red blood cell is typically

around 120 days. Any process that can disrupt the normal life span of a red blood cell may cause anemia. Anemia is caused essentially through two basic pathways: by a decrease in production of red blood cell or hemoglobin, or by a loss or destruction of blood (Firkin *et al.,* 1989).

Owing to the global trend towards improved 'quality of life', there is considerable evidence of an increase in demand for medicinal plant. Use of plants for treating various ailments of both man and animal is as old practice as man himself. India is richly endowed with a wide variety of plants having medicinal value. These plants are widely used by all sections of the society whether directly as folk remedies or indirectly as pharmaceutical preparation of modern medicine. In recent times, focus on plant research has increased all over the world and a large body of evidence has collected to show immense potential of medicinal plants used in various traditional systems (Ayurveda, Siddha and Unani). Medicinal plants are a major source of biodynamic compounds of therapeutic values. (Harsha *et al.,* 2002).

Anemia is one of the numerous ailments claimed to have been successfully treated with plant materials by traditional medicine practitioners. In China for instance, blood diseases such as malformation of blood circulatory system, anemia, varicose veins and haemorrhages have been treated with plant materials (Richard, 1978). A good number of medicinal plants are traditionally employed to alleviate anemia. Sewome of these plants include *Telfeira occidentalis*, *Combretum dolichopetalum*, *Psorospermum ferbrifugum*, *Jatropha curcas, Flacourtia flavenscens* and *Brillantasia nitens* (Alada, 2000; Dina *et al.,* 2006). Keeping this in view, in the present study the chosen plant as *Murraya Koenigii* leaves and evaluate the anti-anemic activity on phenylhydrazine induced anemia in rats.

MATERIALS AND METHODS

Animals

Male albino rats of Wistar strain approximately weighing 180-200g were used in this study. They were healthy animals purchased from the Indian Institute of Science, Bangalore. The animals were housed in spacious polypropylene cages bedded with rice husk. The animal room was well ventilated and maintained under standard experimental conditions (Temperature 27 ± 2º C and 12 hour light/dark cycle) throughout the experimental period. All the animals were fed with standard pellet diet and water were provided *ad libitum*. They were acclimatized to the environment for one week prior to experimental use. The animal feed composition is crude protein (22.3%), crude oil (4.01%), crude fibre (4.02%), Ash (8.02%) and sand silical (1.02%).

Chemicals:

Phenylhydrazine, Sodium hydroxide and Trichloro Acetic acid (TCAs) were purchased for Sigma chemical company, Mumbai All other chemicals and reagents used in this study was of analytical grade with high purity and were obtained from Glaxo laboratories and Sisco Research laboratories, Mumbai, India.

Plant material and preparation of extract

The leaves of *Murraya koenigii* was purchased from local vegetable shop at Thanjavur. The purchased leaves of *Murraya koenigii* were cut into small pieces and shade dried at room temperature and makes a fine powder using grinder mixture. The powder material of *Murraya koenigii* leaves was macerated with 70% ethanol at room temperature for 3 days. After 3 days, the supernatant was transferred into china dish. The supernatant was completely removed by keeping the china dish over a boiling water bath at 45^{o}C. A semi solid extract was obtained after complete elimination of alcohol. The obtained residue was kept in the refrigerator for further use. The extract was made up to a known volume in distilled water just before oral administration.

Experimental Design

Anemia was induced by intraperioneal injection of phenylhydrazine at 40 mg/kg for 2 days (d), as described by Aboudoulatif *et al.,* (2008). Following the injections, rats were divided in four groups of six rats each. The first group, the control received distillated water. The second group received phenylhydrazine only (Anemic group). The third received the *Murraya koenigii* leaves at a dose of 500mg/kg/day and the fourth groups received standard as Hematinic syrup (Dexorange, Franco-Indian Pharmaceuticals Pvt. Limited, Mumbai) at 0.68ml/kg/day respectively. The vehicle, the extract and standard were administered from day 2 to day 15 after phenylhydrazine administration.

Reference hematinic: Dexorange (Dexorange, Franco-Indian Pharmaceuticals Pvt. Limited, Mumbai) was used as a reference hematinic. It is a preparation for treating anemia in children and adults. Each 15 ml of the preparation contains 160 mg ferric ammonium citrate (equivalent 32.8mg elemental iron), vitamin B12 (Cynacobalamin) 7.5mg and Folic acid 0.5mg. The dose of Dexorange administered to experimental animals in this study is equivalent to the adult human dose (0.68ml kg day^{-1}) stated by manufacturers.

Collection of blood sample

At the end of the experimental period, the animals were anaesthetized using chloroform vapour prior to dissection. Blood samples were collected from the caudal vein into a micro centrifuge tube containing 50 mM ethylenediamine tetra acetic acid (EDTA) for the determinations of hematological profile. The blood was collected without EDTA to other test tubes. The blood was allowed to clot by standing at room temperature for 30 minutes and then refrigerated for another 30 minute. The resultant clear part was centrifuged at 3000rpm for 10minutes, and then the serum (supernatant) was isolated and stored at refrigerated until required for analysis.

Biochemical estimations

Malondialdehyde was estimated by the thiobarbituric acid assay method of Beuge and Aust (1978). Reduced glutathione was estimated by method of Moron *et al.,* (1979). Haemoglobin was estimated by Cyanmethaemoglobin method (Dacie and Lewis, 1968) (Beacon Diagnostic Kit). The serum GOT was estimated by the method of Reitman and Frankel (1957) RBC, Platelet and WBC counted by the method of Ochei and Kolhatkar, (2000). PCV counted by the method of Ochei and Kolhatkar, (2000).

The Mean Corpuscular Haemoglobin (MCH)

This indicates the weight of haemoglobin in a single red blood cell and is expressed in pictograms (pg) (1 pg = 10^{-12}g).

$$MCH = \frac{\text{Haemoglobin (g per 100 ml)}}{\text{RBC count million per cu.mm}} \times 10$$

Mean Corpuscular Haemoglobin concentration (MCHC)

This denotes the haemoglobin concentration per 100 ml of packed red blood cells and is related to the colour of the red cells. This is expressed as percentage of packed cells.

$$MCHC = \frac{\text{Haemoglobin (g/d1)}}{\text{PCV \%}} \times 100$$

The Mean Corpuscular Volume (MCV)

This is expressed as the volume in cubic microns or femto liters of an average red blood cell.

$$MCV = \frac{\text{PCV \%}}{\text{Red blood cells in millions per cu.mm}} \times 10$$

Statistical Analysis:

Values were expressed as mean ± SD for six rats in the each group and statistical significant differences between mean values were determined by one way analysis of variance (ANOVA) followed by the Tukey's test for multiple comparisons. The results were statistically analyzed by Graphpad Instat Software (Graphpad Software, San Diego, CA, USA) version 3 was used and $p < 0.01$ and $p < 0.001$ were considered to be significant.

RESULTS AND DISCUSSION

The present study was carried out to evaluate the Anti-anemic activity of *Murraya koenigii* leaves. The observations made on different groups of experimental and control animals were compared as follows. Table 1 represents the levels of MDA and GSH in serum of normal and experimental rats.

Group II phenylhydrazine intoxicated rats showed a significant increased in the level of MDA when compared to Group I rats. Group III phenylhydrazine intoxicated rats treated with *Murraya koenigii* significantly decreased in the level of MDA when compared to group II. Group II phenylhydrazine intoxicated rats showed a significant decreased in the level of GSH when compared to Group I rats. Group III phenylhydrazine intoxicated rats treated with *Murraya koenigii* significantly increased in the level of GSH as compared to groupII.

Table 2 represents the levels of Hb in normal and experimental rats. Group II phenylhydrazine intoxicated rats showed a significant decreased in the content of Hb when compared to Group I rats. Group III phenylhydrazine intoxicated rats treated with *Murraya koenigii* significantly increased in the level of Hb when compared to group II.

Table 1 Effect of *Murraya koenigii* on MDA and GSH in experimental rats

Parameters	Group I	Group II	Group III	Group IV
MDA (nmol MDA formed/l)	3.08 ± 1.32	6.42 ±1.32#	2.99 ± 0.58*	3.45 ± 0.82*
GSH (mg/dl)	7.9 ± 1.83	2.9 ±0.86#	5.04 ±0.75*	5.2 ± 0.62*

Values were expressed as mean ± SD for six rats in each group.
* Significantly different from Group II ($p < 0.001$)
# Significantly different from Group I ($p < 0.001$)

Table 2 Effect of *Murraya koenigii* on Hb content in experimental rats

Parameters	Group I	Group II	Group III	Group IV
Hb (gm/dl)	14.80 ± 3.06	5.96 ± 2.0#	14.82 ± 2.35*	13.93 ± 1.2*

Values were expressed as mean ± SD for six rats in each group.
* Significantly different from Group II ($p < 0.001$)
# Significantly different from Group I ($p < 0.001$)

Table 3 represents the WBC, RBC and Platelet count in normal and experimental rats. Group II phenylhydrazine intoxicated rats showed a significant decreased in WBC when compared to Group I rats. Group III phenylhydrazine intoxicated rats treated with *Murraya koenigii* significantly increased in WBC when compared to group II. Group II phenylhydrazine intoxicated rats showed a significant decreased in RBC when compared to Group I rats. Group III phenylhydrazine intoxicated rats treated with *Murraya koenigii* significantly increased in RBC when compared to group II. Group II phenylhydrazine intoxicated rats showed a significant decreased in Platelet when compared to Group I rats. Group III phenylhydrazine intoxicated rats treated with *Murraya koenigii* significantly increased in Platelet when compared to group II.

Table 3 Effect of *Murraya koenigii* on WBC, RBC and Platelet count in experimental rats

Parameters	Group I	Group II	Group III	Group IV
WBC (cu.mm)	4075 ± 245	2350±236#	3450 ± 278*	3650 ± 290*
RBC (Million/cu.mm)	5.6 ± 0.39	2.6 ±0.18#	4.6 ± 0.24*	3.8 ± 0.20*
Platelet (cu.mm)	3.60000 ± 1744	1,10000 ±2016#	2,90000 ± 1870*	2,22000 ± 0.04*

Values were expressed as mean ± SD for six rats in each group.
* Significantly different from Group II (p< 0.001)
# Significantly different from Group I (p< 0.001)

Table 4 Effect of *Murraya koenigii* on SGOT activity in control andexperimental rats

Parameters	Group I	Group II	Group III	Group IV
SGOT (IU/L)	29.28 ±13.90	90.28 ±22.16#	31.72 ±13.90*	41.24± 16.50*

Values were expressed as mean ± SD for six rats in each group.
* Significantly different from Group II (p< 0.01)
# Significantly different from Group I (p< 0.001)

Table 4 represents the activity of SGOT in normal and experimental rats. Group II phenylhydrazine intoxicated rats showed a significant increased in the activity of SGOT when compared to Group I rats. Group III phenylhydrazine intoxicated rats treated with *Murraya koenigii* significantly decreased in the activity of SGOT when compared to group II.

Table 5 represents the PCV, MCH, MCHC and MCV count in normal and experimental rats. Group II phenylhydrazine intoxicated rats showed a significant decreased in PCV when compared to Group I rats. Group

III phenylhydrazine intoxicated rats treated with *Murraya koenigii* significantly increased in PCV when compared to group II. Group II phenylhydrazine intoxicated rats showed a significant decreased in MCH when compared to Group I rats. Group III phenylhydrazine intoxicated rats treated with *Murraya koenigii* significantly increased in MCH when compared to group II. Group II phenylhydrazine intoxicated rats showed a significant decreased in of MCHC when compared to Group I rats.

Group III phenylhydrazine intoxicated rats treated with *Murraya koenigii* significantly increased in MCHC when compared to group II. Group II phenylhydrazine intoxicated rats showed a significant increased in MCV when compared to Group I rats. Group III phenylhydrazine intoxicated rats treated with *Murraya koenigii* significantly decreased in MCV when compared to group II.

Discussion

Anemia is usually defined as a reduction of the haemoglobin concentration, red blood cell count or packed cell volume to below normal levels. As a result, the oxygen carrying ability of the blood is reduced. The causes of anemia are divided into failure of red cell proliferation, defective maturation of red blood cells, haemolysis, and blood loss. Exogenous agents can induce anemia by some of these different mechanisms. Classification of chemicals that cause anemia by other mechanisms, e.g. blood loss or direct toxicity to the bone marrow resulting in decreased bone marrow production of red blood cells. The exposure to many chemicals including the administration of some drugs has been associated with red blood cell destruction and haemolytic anemia is a part of the clinical syndrome associated with intoxication. Chemicals can cause haemolysis by interacting with sulfhydryl groups, the inhibition of various enzymes, immune mechanisms, and the fragmentation of erythrocytes as they pass through the platelet-fibrin mesh (Beutler, 2001).

Number of chemicals used to induce hemolytic anemia. Among other hemolytic agents, phenylhydrazine is known to induce oxidative damage of both hemoglobin and erythrocyte membrane proteins. Phenylhydrazine (PHZ) is a strong oxidant agent, which is extensively used in industry, laboratory and therapeutic settings. A variety of toxic effects of PHZ have been described, including hemolytic anemia, hypoxia, inflammation, alterations in the liver, kidney, central nervous system, autoimmune disturbances and cancer (Brugnara and de Franceschi, 1993; Goldberg and Stern, 1977).

Table 5 Effect of *Murraya koenigii* on PCV, MCH, MCHC and MCV count in experimental rats

Parameters	Group I	Group II	Group III	Group IV
PCV (%)	48± 3.36	31 ± 2.17#	46 ± 3.22*	41 ± 2.87*
MCH (pg/cell)	26.53 ± 1.87	22.92 ± 1.60#	32.21 ± 2.24*	36.65 ± 2.52*
MCHC (%)	30.83± 2.15	19.22 ± 1.34#	32.21 ± 2.24*	33.97 ± 2.28*
MCV (Cubic micron)	85.71 ± 5.99	119.23 ±8.37#	100 ± 7*	101.89 ±7.49*

Values were expressed as mean ± SD for six rats in each group.
* Significantly different from Group II (p< 0.01)
# Significantly different from Group I (p< 0.01)

This study aimed to evaluate the effect of *Murraya koenigii* on the haemolytic anemia induced by phenylhydrazine in wistar rat. It has been demonstrated previously that intraperitoneal administration of phenylhydrazine decreases haemoglobin concentration, red blood cells number and haematocrit in rat (Diallo *et al*., 2008). This anemia which resulted from the early lysis of the red blood cells was naturally reversed 12 days later by the regeneration of these blood cells due to the increase of the reticulocytes. Our results indicate that the *Murraya koenigii* increases significantly the concentration of haemoglobin, osmotic resistance of red blood cells and the number of reticulocytes, mainly 12 days after phenylhydrazine administration. Moreover, the *Murraya koenigii* is potentiates the increase of the number of reticulocytes. The *Murraya koenigii* could stimulate erythropoisis process. Increase of the number of young red blood cells (reticulocytes) explains the strong osmotic resistance of the red blood cells in rats treated with the extract. The number of circulating reticulocytes coincided with the increase in MCV, thus suggesting that erythrocyte precursors become anucleated at a more differentiated stage of erythropoiesis (Criswell *et al*., 2000). On the other hand, the increase in MCH observed during the experimental period could be indicative of a certain degree of intravascular hemolysis (Criswell *et al.,* 2000).

PHZ is known to shorten life-span of red blood cells (RBCs) resulting in severe hemolytic anemia, enhanced erythropoietic activity, increased iron absorption and tissue iron overload. The auto-oxidation of PHZ leads to generation of reactive oxygen species (ROS) and a complex array of PHZ-derived radicals, such as phenylhydrazyl radical, phenyldiazene and benzenediazonium ions (Misra and Fridovich, 1976). PHZ metabolites can induced oxidative stress on the red cell membrane to cause lipid peroxidation and protein oxidation resulting in the destruction of RBCs and hemolytic anemia. Lipid peroxidation and the resultant perturbation of the structural integrity of the plasma membrane have long been considered to be capable of initiating the hemolytic response, though how generalized destruction of membrane lipids could stimulate a selective macrophage response was not clear. The more recent reports that lipid peroxidation in nucleated cells correlates with accumulation of phosphatidylserine (PS) in the outer leaflet of the lipid bilayer (Tyurina *et al.,* 2004). ROS production was associated with extensive binding of oxidized and denatured haemoglobin to the membrane cytoskeleton. Thus, PHZ-induced haemolytic injury seems to be derived from oxidative alterations to red blood cell membrane lipids (McMillan *et al*., 2005). In the present study, increased lipid peroxidation products as MDA were observed on phenylhydrazine intoxicated rats. Supplementations of *Murraya koenigii* restored the MDA content suggested that reduced the oxidative damage.

Glutathione is a ubiquitous thiol containing tripeptide, which plays a central role in cell biology. It is implicated in the cellular defence against xenobiotics and naturally occurring deleterious compounds, such as free radicals and hydro peroxides. Glutathione status is a highly sensitive indicator of cell functionality and viability. GSH depletion is linked to a number of diseases states including cancer, neurodegenarative diseases, kidney and cardiovascular diseases. Kidneys are exposed to various cytotoxic agents before the elimination of these agents in urine. Thus the GSH concentrations in kidney cells are important (Pastore *et al.,* 2003). In the present study, a marked decrease in the concentration of GSH was observed in phenylhydrazine intoxicated rats when compared to control rats. Administration of *Murraya koenigii* significantly increased in the levels of GSH in phenylhydrazine intoxicated rats.

Significant ($p < 0.001$) decreases in haemoglobin, red blood cell, PCV was observed following injection of the experimental animals with PHZ in this study. The haemolytic activity of arylhydrazine, such as phenylhydrazine (PHZ), dapsone hydroxylamine, divicine, may lead to acute haemolytic anaemia in vertebrates (Ndem, *et al*., 2013). Treatment with *Murraya koenigii* leaf extract increased the concentration of these parameters and even boosted the haemoglobin concentration higher than the positive control animals

PHZ is a well known non immunogenic drug that induces changes in the red cell membrane, which result in oxidative denaturation of haemoglobin. The effect of the denaturation is altered haemoglobin called “Heinz bodies” which reduces the life span of the erythrocytes. This is often characterized by significant increase in the incidence of micronucleated polychromated and hypochromic erythrocytes resulting in increased mean cell volume and decreases mean cell haemoglobin concentration values. Altered erythrocytes are removed by the reticuloendothelial system within the spleen primarily and in the liver to a lesser extent, resulting in a compensated haemolytic anaemia (Ndem, *et al.,* 2013). The following secondary parameters of erythrocytes are often provided to assess the anti-anemic activity of *Murraya koenigii*. The Packed cell volume (PCV: %) often reduced in haemolytic anemia. Mean Corpuscular Haemoglobin Concentration (MCHC): amount of haemoglobin per unit erythrocyte volume: often reduced in haemolytic anemia or increased in case of massive intravascular haemolysis. Mean Corpuscular Volume (MCV) average volume of the erythrocyte: often increased in haemolytic anemia as the result of reticulocytosis. Mean corpuscular haemoglobin (MCH): average amount of haemoglobin per cell: often decreased in haemolytic anemia (Muller *et al.,* 2006).

In the present study, PHZ intoxicated rats decrease Hemoglobin levels, RBC (Red Blood Cell), WBC, platelet count and PCV (Pack Cell Volume), MCH (Mean Cell Hemoglobin) and MCHC (Mean Corpuscular Hemoglobin Concentration)whereas; it induces increase in MCV (Mean Cell Volume). Our results corroborated with earlier reports (Unami, 1996; Ndem, *et al.,* 2013). Supplementation of *Murraya koenigii* to PHZ intoxicated rats restored the altered hematological parameters.

Hepatocellular necrosis/single cell necrosis, inflammation and hepatocytomegaly are also observed in studies on haemolytic substances. The interpretation as to whether these changes are related to haemolysis. Haemosiderin deposition in the liver is a pathological condition. A prominent accumulation of haemosiderin pigment in sinusoidal Kupffer cells and hepatocytes gives an indication of intravascular haemolysis. More subtle lesions such as increases in collagen Wbre production as early indicators of fibrosis, activation of Kupffer cells, transformation of sinusoidal stellate cells or single cell degeneration may be associated with liver haemosiderosis. Macroscopic effects such as increased liver weight, enlarged liver or darkened liver could be indicative of extramedullary haematopoiesis or uptake of residues from haemolysed erythrocytes (Muller *et al.,* 2006). Enzymes catalyze specific biochemical reactions in the body. Changes in their levels and of cellular damage, the intracellular concentration of the enzymes and the mass properties alter the functional ability of an organism. The diagnosis of organ disease/damage is aided by measurement of a number of non-functional plasma enzymes characteristic of that tissue or organ. The amount of enzyme released depends on the degree of affected tissue. The concentration of the enzymes released reflects the severity of the damage. SGOT and SGPT are enzymes normally present in the liver, heart, muscles and blood cells. They are basically located within hepatocytes. So when liver cells are damaged or die transaminases are released into blood stream, where they can be measured they are therefore of index of liver injury (Reitman and Frankel, 1957). The hepatacellular damage indicated by increased the activity of SGOT in serum was observed in this study. Supplementation of *Murraya koenigii* to phenylhyrazine intoxicated rats restored the SGOT activity.

The results of the present study concluded that *Murraya koenigii* inhibits anemia induced by phenylhydrazine, model of anemia similar to those induced by parasite such as *Plasmodium falciparum*. This result supports at least partially the traditional use of *Murraya koenigii* in the treatment of anemia. The potential activity of *Murraya koenigii* leaves which might have been due to the phytochemicals such as flavonoids, steroids, alkaloids and phenolic compounds present in it. Further investigations are needed to understand the mechanism involved in the anti- anemia action of *Murraya koenigii*.

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