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Effect Of Green Gram *(Vigna Radiata)* Dhal Flour Fortified With Iron And Absorption Promoter On The Serum Iron Level Of Female Albino Rat

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ABSTRACT:

Good health depends on good nutrition. Iron Deficiency Anaemia (IDA) is one of the most endangering micro - nutrient malnutrition in India. Mild to severe anaemia not only damage the quality healthy life of the people and their work capacity and productivity. The World Health Organisation (WHO) report already identified Iron Deficiency Anaemia as world's most serious health risk factor. The vulnerable groups are pre-school children, school going children, adolescent girls, pregnant women and partially lactating women. Food fortification is a cost effective process of adding essential micronutrients to food products in order to combat malnutrition. So far, several iron fortification work has already being done with different vehicles like flour, sugar, milk, soya sauce, fish, meat etc. but the concept of fortifying pulse flour is a newer one as amongst different pulses green gram dhal is consumed by many people in India. It has already been established in various in-vitro studies that bioavailability of iron is enhanced when foods are fortified with extrinsic iron, ascorbic acid as iron absorption promoter and Ethylene Diamine Tetra Acetic acid (EDTA) and Sodium Hexa-Meta Phosphate (SHMP) as stabilizer, specially in acidic pH, however, in vivo study with animal model gives more better concept. In the present study green gram dhal flour was considered to be the vehicle of iron fortification in various combinations and those were used to feed female albino rats of same body weight to observe its effect on serum iron and serum ferritin level of those rats. The rats were kept on simple normal diet to accustom with the new laboratory environment and then they were segregated into different groups and put on anaemic diet for 15days to look into the blood parameter changes. They were fed with fortified green gram dhal flour and normal diet for another 15 days. Both the serum iron and serum ferritin levels of female albino rats were decreased than the initial blood level, after getting anaemic diet but they were significantly increased after being treated with the feed of normal diet with fortified green gram dhal flour. Thus it can be predicted that fortified green gram dhal flour has a direct positive impact on the serum iron and serum ferritin level of female albino rats.

Keywords: green gram dhal flour, female albino rat, serum iron, serum ferritin, fortification, ascorbic acid, EDTA, SHMP

INTRODUCTION:

Nutritional iron deficiency is a public health problem in developing countries, including India(Sheshadri,1997). Inadequate intake of iron and consumption of foods low in bio-available iron are identified as the causes of iron deficiency. The World Health Organisation (WHO) report already identified Iron Deficiency Anaemia as world's most serious health risk factor. The vulnerable groups are pre- school children, school going children, adolescent girls, pregnant women and partially lactating women, even in men,in India. In the initial state of Iron Deficiency Anaemia the haemoglobin level in the blood falls below on age-sex specific standard.

Micronutrient Malnutrition (MNM) is pandemic problem and according to WHO, more than two billion people in the world are suffering from MNM amongst which 0.8 million deaths occur every year due to Iron Deficiency Anaemia. "**HIDDEN HUNGER''** is a very contemporary term meaning micronutrient deficiencies. To combat this situation the three way strategic principles are dietary modification, supplementation and food fortification. The approach of enhancing the bioavailability of native food iron seems to be an essential strategy to combat with iron deficiency disorders among the community people, specially in developing countries like India, where poor economic status, ignorance, consumption of nutrient deficient diet, infection, worm infestation etc. directly affect the health of common people.

According to WHO, **"Food fortification is the process whereby nutrients are added to food (in relatively smooth quantities) to maintain or improve the quality of diet of a group, a community or population.''**

The present study green gram dhal flour was considered to be the vehicle of iron fortification in various combinations and those were used to feed female albino rats of same body weight to observe its effect on serum iron and serum ferritin level of those rats. The rats were kept on simple normal diet to accustom with the new laboratory environment and then they were divided into 5groups and put on anaemic diet for 15days followed by feed with fortified green gram dhal flour and normal diet for another 15 days. Any kind of in vivo study with animal model gives more clear view about the changes of blood parameter and here also it was helpful and useful enough to understand the changes among the albino rats of different groups.

OBJECTIVE:

The present study has been undertaken with the objective of evaluating the in-vivo experiment with female albino rats and to observe the changes in their serum iron status, if any, with various combination of fortified green gram dhal flour.

LITERATURE REVIEW:

The minerals present in the human body in less than 0.05%, are defined as **Microminerals or Trace elements**. Iron is one of the important micromineral that determines the good health of every human being. Iron was first recognized as a constitute of body by Lemory in 1713.

DISTRIBUTION OF IRON IN HUMAN BODY:

SL. No.	Types of Iron	SL.no.	Body Parts	Percentage(%)
1	Functional	1.1	Haemoglobin	60-70
		1.2	Myoglobin	3-4
		1.3	Tissue Iron(enzyme)	5-15
2	Storage and Transport	2.1	Storage iron(liver, spleen, bone marrow)	15-30
		2.2	Transport Iron as Transferrin	0.10
		2.3	Serum Ferritin	<1

[Guthrie Helen A., Marry F. Picciano, Human Nutrition, McGraw-Hill,Boston,1999]

The eventual consequences of iron deficiencies are IRON DEFICIENCY ANAEMIA (IDA) where the *body's store of iron has been depleted and the body is unable to maintain levels of haemoglobin in the blood.* Children and pre-menopausal women are the most vulnerable groups, however iron deficiency anaemia is also found in men, in India.

The various symptoms of iron deficiency include, tiredness, lethargy or lack of energy, shortness of breath(dyspnoea), impaired thermoregulation,Immune dysfunction, GI disturbances, neuro-cognitive impairment, chronic kidney disease (if not treated on time), congestive cardiac failure, chronic respiratory distress (in children)

Less common symptoms include- headache, altered sense of taste, sore tongue, Pica- a desire to eat non-food items, such as ice, paper, mud etc, Tinnitus – perception of noise in one or both ears or in the head that comes from inside the body, ringing of ears, feeling itchy. (Srilakshmi B.,2008)

PREVALANCE OF IRON DEFIENCY ANAEMIA

Anaemia, defined as low blood haemoglobin (Hb) concentration, is a serious global health problem and it relates to negative health outcomes. The World Health Organization(WHO) projects that almost two billion people or 25% of the world's community are anaemic, with roughly half of thgem having IDA.

ANAEMIA PREVALENCE IN CHILDRE , NFHS-5(2019-2021)

Anaemia in children-

Anaemia Status	***Haemoglobin level in gm/dL***
Anaemic	*<11.0*
Mildly anaemic	*10.0-10.9*
Moderately anaemic	*7.0-9.9*
Severely anaemic	*<7.0*
Non anaemic	*11.0 or higher*

***Haemoglobin levels are adjusted for altitude in enumeration areas that are above 1000 meters.*

Sample: children 6-59 months

ANAEMIA PREVALENCE AMONG WOMEN AND MEN, NFHS-5(2019-2021)

Haemoglobin levels below which women and men are considered anaemic-

Respondents	***Haemoglobin level in gm/dL***
Non-pregnant women age 15-49	*<11.0*
Pregnant women age 15-49	*<12.0*
Men age 15-49	*<13.0*

**haemoglobin levels are adjusted for smoking, and for altitude in enumeration areas that are above 1000 meters.*

ROLE OF IRON IN HUMAN BODY

I. ***Transport and storage of oxygen:*** Iron present within Hemoglobin (pigment of red blood cells) and myoglobin. It binds to the oxygen and facilitates its movement from the lungs through the arteries to the cells throughout the body. Once oxygen is delivered, the iron (as a part of haemoglobin) binds the carbon dioxide which is then transported back to the lungs from where it gets exhaled. Myoglobin is found only in the muscles. There is acts as a reservoir of oxygen which is needed to produce the energy for muscle contractions.

II. ***Cofactor for enzymes:*** The iron containing haem group is a part of several proteins involved in the release of energy during oxidation of nutrients and formation of energy rich compounds (ATP). Also iron can itself act as a co-factor for different enzymes in the body.

III. ***Formation of Red Blood Cells (RBC):*** Bone marrow produces erythroblasts. As it matures iron is required along with vitamin B6 and copper.

Iron loss: In an adult, basal iron loses from the gastro intestinal tract, skin, urinary tract is 1mg/day to 14mcg/kg/day. In women of child bearing state, the losses due to menstruation must be added to the basal iron losses that is equivalent to 15mcg/kg/day, thus when the basal losses of iron is added to menstrual losses, the ceiling iron loss at that time in a woman body reaches up to 30mcg/kg/day, if the body weight is 55kg. ***Iron balance:*** Iron balance implies that there is an equilibrium between the absorption and losses of iron from the body. The total amount of iron in the body is by definition constant. Both the absorption and the losses are related to the total amount of iron in the body and they are balanced by ***Redistribution of iron between the compartments and Recycling of iron in the body.***

Haem iron absorption- it consists primarily of haemoglobin and myoglobin. It represents small fraction of the iron in the diet but with high biological value and absorption. Most of the haem iron seems to enter the intestinal absorptive cell as an intact metalloporphyrin. Subsequently iron is released from the porphyrin in the intestinal mucosa by mucosal haem oxygenase and enters the circulation as metallic iron.

Non-haem iron absorption- It is found typically in cereal pulse based diet and also available from green leafy vegetables. Non-haem iron absorption occurs mostly in the proximal small intestine. (UNICEF report2011, Srilakshmi B.,2008)In both the cases, ascorbic acid plays a key role in accelerating iron absorption in human body, specially from non-haem iron, converting the ferric form to absorbable ferrous form.

THE MAIN WAYS THAT PEOPLE GETS MICRONUTRIENTS:-

- Diverse diet high in high fruits, vegetables, whole grains and protein rich animal product.
- Supplementation – taking a vitamin / minerasl tablet or pill.
- Bio-fortification foods that are engineered to have higher amounts of certain nutrients,
- Food fortification – addition of micronutrients to food product already consumed.

THE OVERALL EFFECT OF FOOD FORTIFICATION

- Food fortification not only helps to improve overall nutritional status of a population. It also lead to an improved economy. Deficiency infections and disease rate all drop, leading to a decreased mortality.
- Typically socially acceptable, it requires no change in food habits and can be introduced quickly and effectively.
- Today, foods and beverages with added nutritional benefits are great option to help people meet their daily vitamin and mineral needs. This is especially true in two instances :-
  - People with poor appetite who are eating very little and have low nutrient consumption.
  - Athletes with very high needs, who are unable to get enough nutrient through regular food.

Animal studies :- in the 1970s and 80s, many iron bioavailability studies on different food components were performed like in rats, chick even pigs, using both radiosotopes and the haemoglobin repletion methodologies (Pla & Fritz, 1971).

The steps of animal study involves the following-

- Animal selection

- Diet preparation
- Feeding and design
- Iron absorption
- Iron retention
- Iron utilization

INVIVO in Latin means "within the living". In any in vivo experiment, scientists usually conduct their studies in whole living organism. It is generally better for the researchers to be able to observe overall effects of scientific research on living subjects in the natural and desired environment, however as it deals with living subjects it becomes challenging too to maintain them for the entire duration of research work.

METHODOLOGY:

Green gram dhal flour, Ferrous sulphate heptahydrate (FeSO4,7H2O), Ascorbic acid, NaEDTA ,SHMP were used for the study. The dhal flour was procured locally and after ensuring the fact that it was free from contaminant, it was processed for fortification by washing, drying, milling respectively. Ferrous sulphate salt, a cost effective source of iron, L-ascorbic acid, EDTA, SHMP were of analytical grade and procured locally.

According to various studies, it was found that Indian adolescents girls are one of the vulnerable group suffering from Iron Deficiency Anaemia (IDA), hence the present study considered the Recommended Dietary Allowance (RDA) of adolescents girls of 16-18 years i.e. 32mg/day (ICMR 2020) to derive the level of fortification. Usually, fortification should 1/3rd of the RDA, hence it was almost equal to *10 mg/day.* Green gram dhal flour has 4mg intrinsic iron/100gm. Considering the acceptable edible quantity of dhal flour as 50gm/day by any human being, the available intrinsic iron would be 2mg/100gm and rest part can be fortified with extrinsic iron.

With the calculated amount of extrinsic iron as FeSo4,7H2O ascorbic acid as iron absorption promoters, EDTA and SHMP as stabilizer the following combinations were prepared with green gram dhal flour for the study:

1. *Green Gram dhal flour +no fortificant (control)*
2. *Green Gram dhal flour + $FeSO_4, 7H_2O$*
3. *Green Gram dhal flour + $FeSO_4, 7H_2O$ + Ascorbic acid*
4. *Green Gram dhal flour + $FeSO_4, 7H_2O$ +EDTA*
5. *Green Gram dhal flour + $FeSO_4, 7H_2O$ +SHMP*

A five time concentrated premix of each of the fortificants was prepared in green gram dhal flour base. A two-stage dry mixing (hand procedure) was adopted to obtain a homogenous preparation and then it was diluted by mixing the vehicle in the desired amount.

Iron content of all the preparations were in the mineral solution of the dry digested samples according to Wongs method. About 5-10gm of control and fortified green gram dhal flour were ashed at 600degree Centigrade in a muffle furnace for 12 hours. The residues were treated with concentrated nitric acid and hydrochloric acid and evaporated to dryness. the residue thus obtained was dissolved in 5ml 6N HCL and filtered and process was repeated for 2-3 times with glass distilled water. The combined filtrate were made upto 100ml and further steps were followed as described in Wongs method. (Nayak B., Nair K.M.2003)

The study was designed to evaluate the blood parameters of female albino rats by in vivo method as blood parameters of albino rat gives almost same indication of the changes as in case of blood parameters of human. 25 female albino rats (non pregnant) with average body weight of 100-120gm were used for this study. At first they were given normal diet for 7 days to adapt with the new laboratory environment and initial blood samples were taken to check the required blood parameters. They were then put into anaemic diet for 15 days to see

whether there would be any drop in the serum iron level or not. After that the rats were segregated into five groups and also divided into control group and test groups. Each of the group was fed with normal diet along with fortified green gram dhal flour in various combinations.

In this study no commercial preparation was used as sample rat diet, rather focus was given more on preparing normal diet for all the rats with natural ingredients so that the anaemic diet can also be prepared with natural ingredients and it would give a symmetry in the study.

Usually an adult rat consume around 12-15gm food/day (Nutrient requirement of Laboratory Animals,1995). This study was consisted of 25 rats so an average of 400gm food mix with natural resources was required and depending on the percentage of various nutrients in a normal rat diet(Nair. K. M.2003) the composition was as follows:

For each 400gm food mix:

1.	Wheat flour	60 gm
2.	Roasted Bengal gram flour	232 gm
3.	Ground nut flour	40gm
4.	Skimmed milk powder	20 gm
5.	Casein powder	16gm
6.	Refined oil	16ml
7.	Salt	16gm

It was calculated that the iron content of the normal diet prepared for rat was 6.295mg/100gm and hence each rat would get around 0.944mg iron/day. While preparing the anaemic diet the roasted Bengal gram flour was replaced with corn flour and the iron content of that diet dropped to 1.365mg/100gm and thus 0.204mg iron/rat/day. The body weight was measured regularly for each rat. The tail cut blood was collected from each sample rat at the initial stage and after giving anaemic diet, however the final blood sample was collected by dissection of rats after completion of the study.

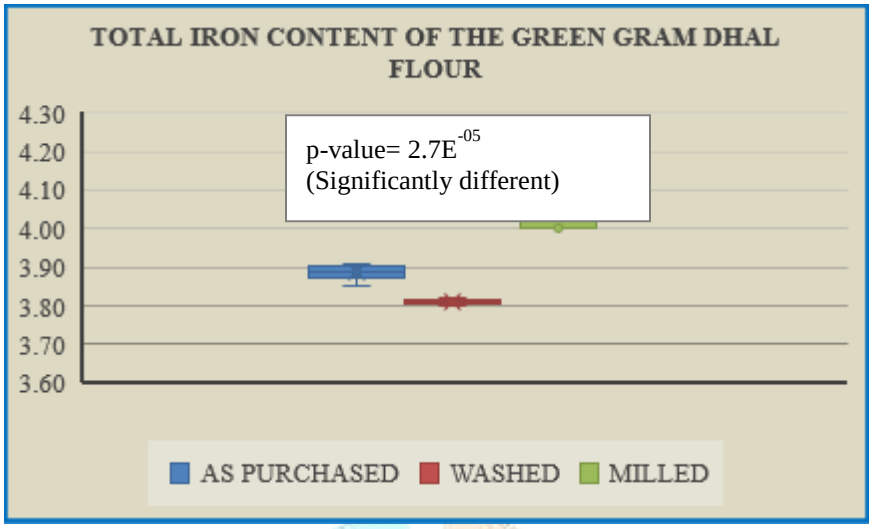
RESULT:

The t-test, also known as Student's T test and one way ANOVA were used for statistical analysis. The Null Hypothesis was considered as there were no differences between the groups. T-Test provide p-value based on t-distribution and if the p-value is less than the chosen significance level (0.05), the null hypothesis is rejected and the result was considered statistically significantly different as per the alternative hypothesis.

The study also represents the data in a Box and Whisker Plots and other diagrams to get a snapshot of the data and analysis at a glance. Box plots are used to show distributions of numeric data values, especially when we want to compare them between multiple groups. They are built to provide high-level information at a glance, offering general information about a group of data's symmetry, skew, variance, and outliers.

The iron content of each of these combinations were estimated and the were as follows:

Total Iron content of the Green Gram Dhal Flour as purchased, after washing and after milling



TOTAL IRON CONTENT OF PREMIXES, FORTIFIED GREEN GRAM DHAL FLOUR

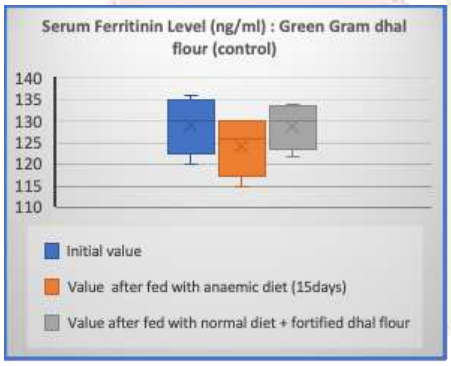
Combinations of Research sample	**Iron content of Premix (observed)**	**Iron content of Fortified Dhal flour(observed)**
	Mean±S.D.	Mean±S.D.
Green Gram dhal flour (control) – No fortificant	4.04±0.049	4.01±0.007
Green Gram dhal flour + $FeSO_4$, $7H_2O$	35.06±0.040	9.40±0.005
Green Gram dhal flour + $FeSO_4$, H_2O + Ascorbic acid	33.19±0.024	10.12±0.075
Green Gram dhal flour + $FeSO_4$, H_2O + EDTA	32.16±0.019	9.6±0.013
Green Gram dhal flour + $FeSO_4$, H_2O + SHMP	35.72±0.075	10.6±0.063

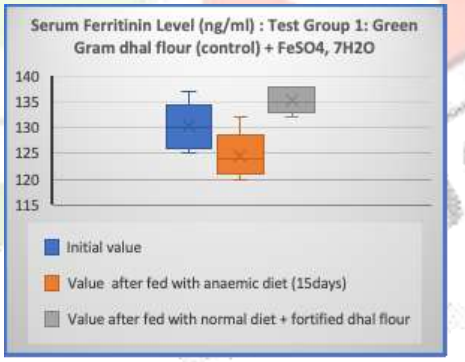
N=5 for each group

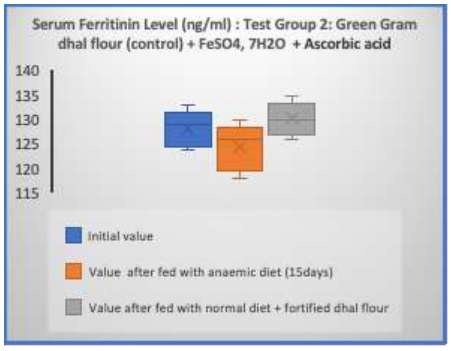
Table 1: ESTIMATION OF SERUM FERRITIN LEVEL OF FEMALE ALBINO RATS AT INITIAL STAGE, AFTER PROVIDING ANAEMIC DIET AND AFTER GETTING FORTIFIED DHAL FLOUR WITH NORMAL DIET:

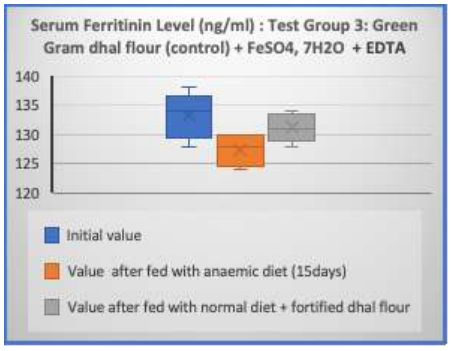
Combinations of Research	**INITIAL VALUE**	**Value after fed with anaemic diet (15days)**	**Value after fed with normal diet + fortified dhal flour**
	Mean±S.D.	Mean±S.D.	Mean±S.D.
Green Gram dhal flour (control)	129.0±5.865	124.2±5.879	128.8±4.622
Test Group 1: Green Gram dhal flour + $FeSO_4$, $7H_2O$	130.2±4.167	124.6±4.079	135.4±2.332
Test Group 2: Green Gram dhal flour + $FeSO_4$, H_2O + Ascorbic acid	128.2±3.311	124.4±4.317	130.2±3.124
Test Group 3: Green Gram dhal flour + $FeSO_4$, H_2O + EDTA	133.2±3.429	127.4±2.498	131.2±2.135
Test Group 4: Green Gram dhal flour + $FeSO_4$, H_2O + SHMP	131.2±4.400	122.6±3.137	127.2±1.720

N=5 for each group









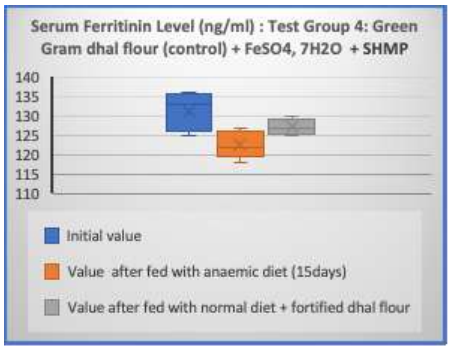
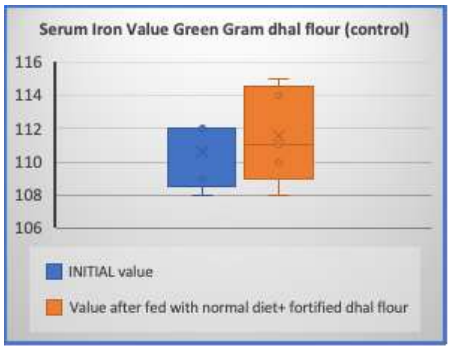
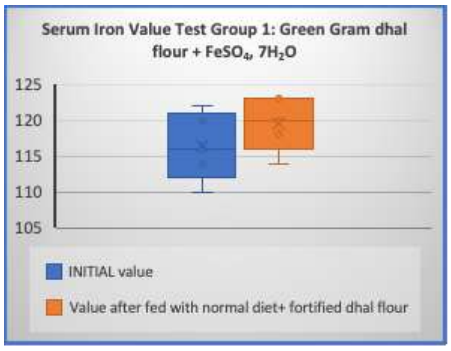


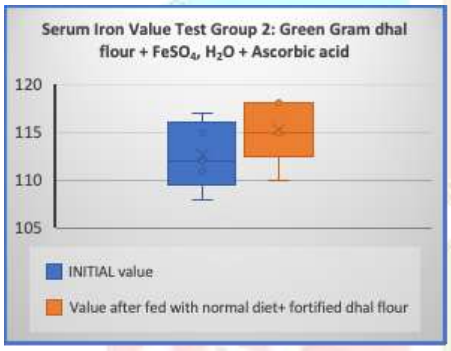
Table 2: ESTIMATION OF SERUM IRON LEVEL OF FEMALE ALBINO RATS AT INITIAL STAGE AND AFTER GETTING FORTIFIED DHAL FLOUR WITH NORMAL DIET:

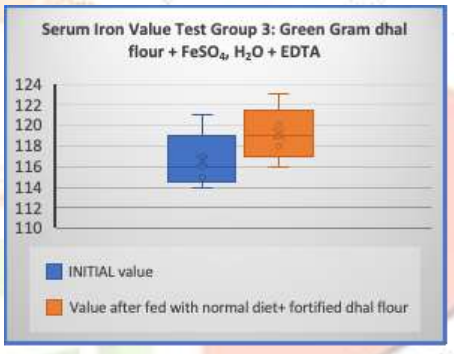
Combinations of Research	**INITIAL VALUE**	**Value after fed with normal diet**
	Mean±S.D.	Mean±S.D.
Green Gram dhal flour (control)	112±1.744	112±2.577
Test Group 1: Green Gram dhal flour + $FeSO_4$, $7H_2O$	116±4.271	119±3.382
Test Group 2: Green Gram dhal flour + $FeSO_4$, H_2O + Ascorbic acid	113±3.137	115±2.926
Test Group 3: Green Gram dhal flour + $FeSO_4$, H_2O + EDTA	117±2.417	119±2.315
Test Group 4: Green Gram dhal flour + $FeSO_4$, H_2O + SHMP	115±4.587	117±3.441

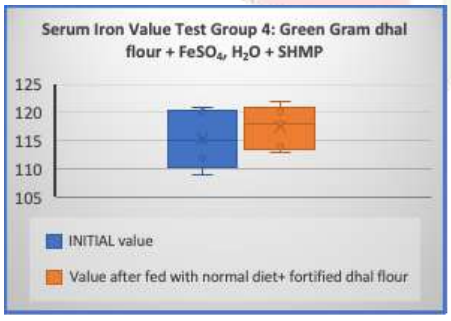
N=5 for each group











As the result signifies, both the serum ferritin level and serum iron level were increased in case of test groups who were fed on fortified green gram dhal flour in different combinations, but the value of control group remain unchanged. It was also noticed that when the rats were on anaemic diet the blood parameters dropped down in all the cases but it was elevated after treated with fortified feed in combination with normal diet consisted of natural ingredients.

DISCUSSION:

The statistical analysis showed that there was no change in the intrinsic iron content of green gram dhal flour as purchased and after washing but a significant change was found after milling of the flour, probably because of the use of iron body mortar.

In case of iron content of fortified green gram dhal flour premix and diluted fortified flour, there were no change in the control portion but iron content of premix was desirably high as compared to the diluted fortified dhal flour.

After the statistical analysis it was found that serum ferritin level was decreased for each group of rat sample after getting the anaemic diet but it was elevated when they were fed with normal diet and fortified green gram dhal flour in graded combination. There was no significant difference in the value of control group but there was remarkable elevation in the serum ferritin level in the test group samples, however the best result was obtained with the fortification combination of *Green Gram dhal flour + $FeSO_4$, $7H_2O$* and *Green Gram dhal flour + $FeSO_4$, $7H_2O$ +SHMP*. In case of serum iron level no change was found in the control group between initial blood result and final blood result as they were not on fortified food but on normal diet only. But significant changes were observed among the test groups treated with various combinations of fortified green gram dhal flour and the final serum iron value was elevated as compared to the initial value before treatment. The two best combinations giving higher serum iron level in the rat blood sample were *Green Gram dhal flour + $FeSO_4$, $7H_2O$ and Green Gram dhal flour + $FeSO_4$, $7H_2O$ + Ascorbic acid.* Thus from the above study it can be predicted that female albino rats when treated with green gram dhal flour fortified with iron and its absorption promoter ascorbic acid and also the stabilizer EDTA & SHMP has a positive elevation effect on their serum iron and serum ferritin level.

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