

Effect of Aqueous Extract of *Glycine max* on Some Iron Profile Parameters in Female Albino Wistar Rats.

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ABSTRACT

Objective/Purpose: The effect of aqueous extract of soybean (*Glycine max*) on serum iron, serum hepcidin level, serum ferritin, erythroferrone, total iron binding capacity (TIBC) and haematological parameters in female albino wistar rat was evaluated. **Materials and Methods:** Thirty (30) female adult rats were randomly selected into three groups containing 10 rats each. Aqueous extract of *Glycine max* was prepared and two different concentrations (1000mg/kg and 2500mg/kg) were administered for 21 days to female albino wistar rats. Serum erythroferrone, serum ferritin, total iron binding capacity (TIBC), serum iron and serum hepcidin were estimated using Enzyme Linked Immunosorbent Assay (ELISA). Full blood count was assayed using Mythic 18 haematology analyzer. Independent t test was used to compare between the control group and the test group, while ANOVA was used to compare between the different concentrations of the extract and the control group and result reported in mean±SEM. **Results:** The results showed a significant increase ($P=0.022$) in total iron binding capacity in the experimental group (5696 ± 200.7) when compared to controls (3051 ± 430.9). Similarly, there was a significant decrease ($P=0.04$) in hepcidin in the experimental group (494.5 ± 32.82) when compared to controls (652.2 ± 58.85). The mean erythroferrone concentration was significantly higher ($P=0.023$) among the experimental group (724.5 ± 18.20) when compared to controls (657.2 ± 14.78). Serum ferritin was also significantly higher ($P = 0.015$) in the experimental group (1.524 ± 0.2334) when compared to controls (0.7843 ± 0.2444). TIBC and serum erythroferrone were significant higher ($p<0.001$) in experimental group C (2500mg/kg) and this appear to be dose dependent. **Conclusion:** The results obtained shows that aqueous extract of soybean (*Glycine max*) increases total iron binding capacity, erythroferrone and ferritin levels and decreases hepcidin levels in female albino Wistar rats.

Keywords: Soybean, hepcidin, erythroferrone, ferritin, serum iron.

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INTRODUCTION

Soybean (*Glycine max*) is an important legume grown in tropical, subtropical and temperate climates. Like peas, beans and peanuts, it belongs to the botanical family, Leguminosae, in the subfamily Papilionideae (1). Soybean serves as source of food and feed for human and animals. Soybean is a concentrated source of isoflavones (2). Soybeans are among food rich in total phytoestrogens which are present in the form of isoflavones, daidzein and genistein (3). Soybeans also have a high iron and ferritin content (4). Yang *et al.* (5), postulated some possible mechanism by which estradiol could increase iron absorption and iron release from storage cells. Their study described a functional estrogen response element (ERE) half-life in the human hepcidin promoter, and they observed that after estradiol treatment, hepcidin mRNA were decreased both *in vitro* study (human liver cells) and *in vivo* study (in mice). In addition, phytoestrogens has been reported to increase estrogen concentration and androgen has been reported to stimulate erythropoiesis, through the conversion to estrogen (6). Iron is an essential trace metal involved in oxygen transport, cellular metabolism, DNA synthesis, innate immunity, growth, and development (7). The diagnosis of iron deficiency usually involves the measurement of serum iron and serum ferritin concentration which is the measure for iron store and it's the most cost effective and efficient test for iron deficiency (8). Serum ferritin decreases during the first stage of iron depletion and can identify low iron status before the onset of iron deficiency anemia (9). Hepcidin is the master regulator of iron metabolism in humans by controlling the absorption of iron, through its action of ferroportin (10). Mammalian iron homeostasis is concertedly regulated through hepcidin and ferroportin that fundamentally govern iron absorption, transport, storage,

and utilization (11). The endocrine system plays a major role in the regulation of erythropoiesis and this may in part be through the direct effect of estrogen on hepcidin and erythroferrone (12). The levels of serum transferrin and total iron binding capacity (TIBC) can be affected by hepcidin, which may lead to functional iron deficiency and anaemia (13). This study determined the effect of soybeans (*Glycine max*) on the level of hepcidin, serum ferritin, erythroferrone, total iron binding capacity (TIBC), some haematological parameters and serum iron.

MATERIALS AND METHODS

Identification of the Soybean

Soybeans (*Glycine max*) of weight 7.5kg was bought at market, located along Uselu-Lagos road, in Egor Local Government of Edo State. It was further transported to the Department of Plant Biology and Biotechnology, Faculty of Physical Sciences, University of Benin, for authentication and given a voucher number UBH-G532.

Method of Extraction of Soybean

Soybean (*Glycine max*) processing was carried out in Trigas Research Laboratory at the Department of Medical Biochemistry, School of Basic Medical Sciences, University of Benin. The seeds were air dried for a period of five (5) days in a cool temperature environment. The seeds were pulverized. The weight was recorded as 3.5 kg. Three (3) kg of Soybeans was macerated in 4 litres of distilled water for 48hrs. It was stirred at intervals. The liquid-soaked sample was filtered initially with muslin cloth and then with Whatman filter paper, a transparent solution of 13 litres was obtained. Four (4) litres of aqueous filtrates were next placed in a freeze dryer for the solvent to evaporate daily until dried extracts are obtained. 548g of dried extracts were obtained and

transferred into vials and stored at 4°C for analysis.

Ethical Approval

The protocol for this study was approved by the Ethical Clearance Committee, Edo State Ministry of Health with the reference number HA. 737/66 dated 9th March, 2021.

Experimental Design and Animal Care

Thirty (30) female adult wistar rats were purchased from the animal holdings of the Department of Anatomy, University of Benin, Benin City and housed in a cross ventilated room in the animal holdings of the Department of Anatomy, University of Benin, Benin City. The rats were acclimatized for a period of two weeks and fed using Grower's mash for five weeks (acclimatization and administration). The extract was administered to rats for 21 days (three weeks). Thirty (30) female adult wistar rats were randomly selected into a control group (group A) and experiment group. The experimental group was further subdivided into 2 groups (B and C) containing ten (10) animals each ($n=10$ per group) to determine if change associated with soybean consumption were dose dependent. The animals were cared for according to the guidelines of the National Institute of Health (NIH, USA). The animals in each cage were given Growers' mash and water daily. The rats were acclimatized for a period of two week and subsequent administration of the aqueous extract of soybeans through galvage to the treatment group (B and C): Group B (1000mg/kg) and Group C (2500mg/kg) was done for 21 days. Control Group (Group A) wistar rats were fed on Grower's mash and water only. The volume (w/v) of extract administered to each rat were calculated from the weight of each rat and the dosage given in

mg/kg. The LD₅₀ have previously been reported to be greater than 5000 mg/kg (14).

Sacrifice of the Animals

At the end of the experimental period, the animals were grossly observed for general physical characteristics. A midline incision was made through the ventral wall of the rats under mild anaesthesia using chloroform. Five milliliters (5ml) of blood were collected from each rat and placed in the EDTA bottles and plain containers. Samples were stored in refrigerator at 4°C.

Preparation of Samples for Biochemical Assay

Serum Preparation

Three milliliters (3mL) of whole blood was collected and allowed to clot by leaving it undisturbed at room temperature. The clot was then removed by centrifuging at 2,000 rpm for 15 minutes and the serum decanted and stored at -20°C.

Laboratory Analysis

ELISA was used to assess the concentration of serum hepcidin, serum erythroferrone, and serum ferritin. Serum iron and UIBC were assayed colorimetrically. TIBC was then calculated from values of serum iron and UIBC.

Determination of Serum Hepcidin

Wells for diluted standard, blank and sample were labeled for analysis. Forty microliter (40µl) of sample dilution buffer was added to 10µl of sample (dilution factor is 5). Samples were then loaded onto the bottom without touching the well wall and mixed with gentle shaking. The strip plate well was incubated for 30 mins at 37°C and sealed with closure plate membrane. The closure plate membrane was carefully peeled off, aspirated, and refilled with the wash solution for 5 times. To

each well, 50 μ l HRP - conjugate reagent was added except the blank control well. The strip plate well was incubated for another 30 mins at 37°C and sealed with closure plate membrane. The closure plate membrane was carefully peeled off, aspirated and washed another 5 times. To each well, 50 μ l chromogen solution A and 50 μ l chromogen solution B were added and mixed by gently shaking and incubated at 37°C for another 15 minutes. To each well, 50 μ l stop solution was added to terminate the reaction. The absorbance O.D was read at 450nm.

Determination of Serum Erythroferrone

To each well, 100 μ l of the different standards was added in duplicate. At the same time, 100 μ l of diluted serum samples were added in duplicate to the wells. The plate was covered with plastic film and incubated for 2 hours at room temperature. The coated wells were aspirated and 300 μ l of washed using a wash buffer as described by the manufacturers. To each well, 100 μ l of the diluted detection antibody (DET) was added. The plate was covered with plastic film and incubated for 1 hour at room temperature. The coated wells were washed a second time following manufacturer's instructions. Hundred (100) μ l of the diluted HRP Labelled streptavidin (STREP-HRP) was added to each well. The plate was covered with plastic film and incubated for another 30 minutes at room temperature. And the well plates further washed. To each well, 100 μ l of TMB substrate solution was added and the reaction was stopped by adding 100 μ l of stop solution (STOP). The plate was tapped gently to ensure thorough mixing. The OD was then read at 450nM

Determination of Serum Ferritin

To each assigned microtitre well, 25 μ l of control and test specimen was added. To each well, 100 μ l of the ferritin biotin reagent was also added. The micro plate was swirled for 20secs, covered and incubated for 30 minutes at room temperature. The content of the micro plate was decanted and a paper was used to blot the micro plate dry. The microplate wells were then washed 3 times following manufacturer's instructions. To each well, 100 μ l of ferritin enzyme conjugate was added. The plate wells were further washed 3 times. Hundred (100) μ l of working substrate solution was then added to each well. The micro plate wells were then incubated for 15 minutes at room temperature. To each well, 50 μ l of stop solution was added, the micro plate was mixed gently for 20secs and absorbance read at 450 nm wavelength.

Determination of Serum Iron

Tubes were appropriately labelled to reflect the control, test and blank, then 2.5ml iron buffer reagent was added to control and test tubes. Sample (0.5ml) was then added to each tube for test and 0.05ml iron-free water was added to blank. The samples were then read at 560nm wavelength. The absorbance of all tubes (A1 reading) were read and recorded. After recording A1 reading, 0.05ml of iron colour reagent was then added to all tubes. The solution was mixed and incubated at 37°C for 10 minutes. The absorbance was read at 560nm against reagent blank. The absorbance of all tubes were read (A2 reading) and recorded. Then A1 absorbance was then subtracted from A2 absorbance, and concentration calculated from the final absorbance.

UIBC (Unsaturated iron-binding Capacity) and Total Iron Binding Capacity (TIBC) Evaluation.

Tubes were appropriately labelled to reflect the control, test and blank, 2.0ml UIBC buffer reagent was added to each tubes. Iron-free water (0.5ml) and 0.5ml of standard was added to the standard, while 0.5ml of sample and iron standard was added to the tube labeled test. Then 1.0ml iron-free water was added to the blank. The spectrophotometer was zeroed with the blank and read at 560nm wavelength. The absorbance of all the tubes (A1 reading) was read and recorded. 0.05ml of iron colour reagent was added to all tubes. The solution was mixed and placed in water bath at 37°C for 10 minutes. The spectrophotometer was zeroed using the blank at 560nm wavelength. The absorbance of all the tubes were read (A2 reading) and recorded. Then A1 absorbance was then subtracted from A2 absorbance, and concentration calculated from the final absorbance.

Total Iron Binding Capacity (TIBC) was calculated thus:

Total Iron Binding Capacity (TIBC) ($\mu\text{g/dl}$)
= Iron level + UIBC

FULL BLOOD COUNT (FBC)

Method: Automation using Mythic 18 haematology analyzer

Statistical Analysis

Statistical analyses including descriptive statistics was carried out using the Graphpad prism version 8.0 California, USA. All values were expressed as mean \pm standard error of mean (SEM). The Independent t-test was used to determine significant differences in test parameters in the experimental group and control group. Analysis of variance

(ANOVA) and turkey's Post Hoc test were used when there was a difference between 2 or more groups. Confidence limit was set at 95%; level of significance ($p < 0.05$).

RESULTS

The results show the effects of soybean diet on serum iron concentration, hepcidin level, total iron binding capacity, erythroferrone, serum ferritin, and haematological parameters. Of the thirty (30) wistar rats used for this study, 10 female rats were used for control (group A), while 20 were test group (female rats). The experiment group were further divided into group B (1000mg/kg body weight) and group C (2500mg/kg body weight).

Figure 1 shows the effect of aqueous extract of soybean (*Glycine max*) on serum iron concentration, TIBC and serum ferritin. There was no significant difference ($p=0.575$) in serum iron levels in the experimental group when compared to controls as shown in panel A. Total iron binding capacity (TIBC) and serum ferritin were significantly higher ($p < 0.001$) in the experimental group when compared to controls (group A) as shown in panel B and C of figure 1.

Figure 2 shows the effect of *Glycine max* on hepcidin and erythroferrone. Panel A shows that hepcidin was significant lower ($p=0.017$) in the experimental group (494.5 ± 32.82) when compared to controls (group A) (652.2 ± 58.85). Panel B also shows that erythroferrone was significantly lower ($p < 0.001$) in the control (group A) (657.2 ± 14.78) when compared to experimental group (724.5 ± 18.20).

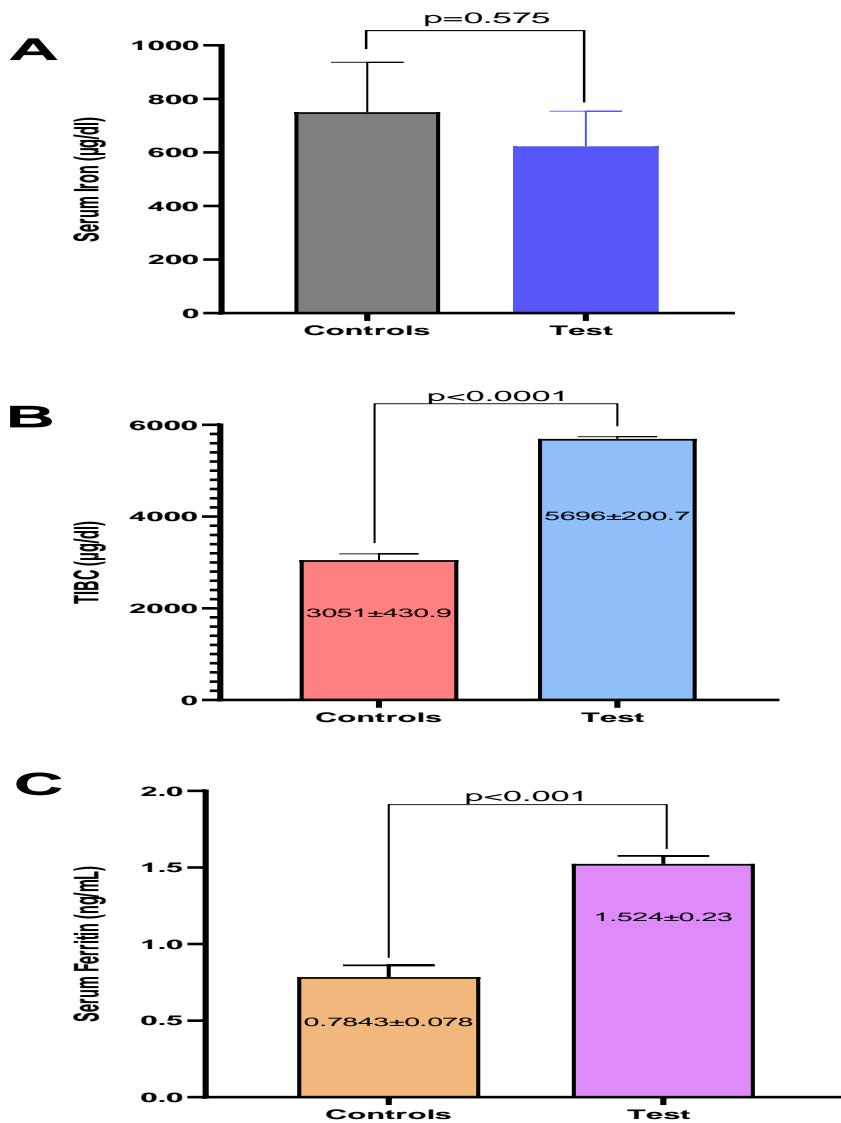


Figure 1: Panel A, B and C shows the effect of soy bean on serum iron, TIBC and serum ferritin in experimental group compared to control. Error bar in all panels showing standard error of mean

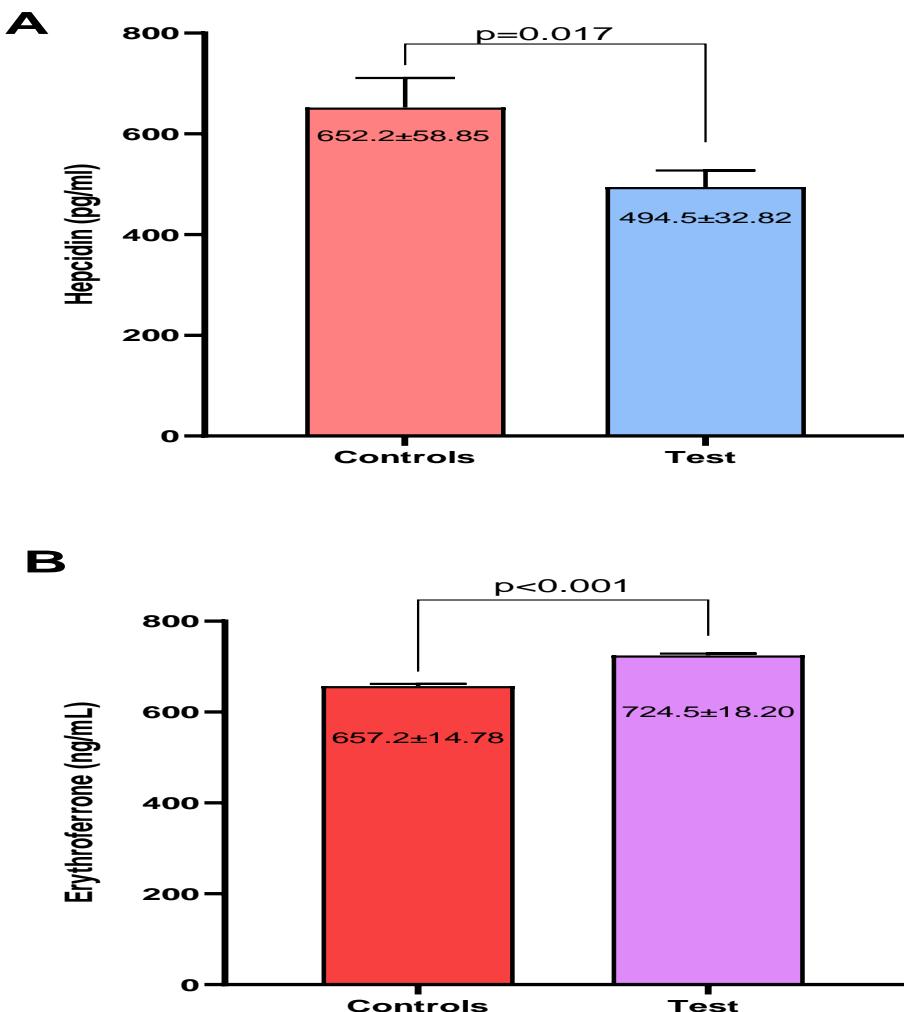


Figure 2: Panel A and B shows the effect of soy bean on hepcidin and erythroferrone concentrations in experimental group compared to control. Error bar in all panels showing standard error of mean

When comparing the effect of 2 different concentrations of *Glycine max* on some iron homeostatic parameters measured. Figure 3 panel A shows that in serum iron, there was no significant difference ($p>0.05$) between the control (group A), group B (1000mg/kg body weight) and group C (2500mg/kg body weight). Panel B of figure 3 shows a significant increase ($p<0.001$) in TIBC in group B (4273 ± 240.7) and group C

(5076 ± 269.9) when compared to control (group A) (3051 ± 430.9). Furthermore, TIBC in group C was also significantly higher ($p<0.001$) when compared to group B. In addition, panel C showed that serum ferritin was significantly higher ($p<0.001$) in group B (1.422 ± 0.1) and group C (1.642 ± 0.076) when compared to control (group A) (0.7843 ± 0.07729).

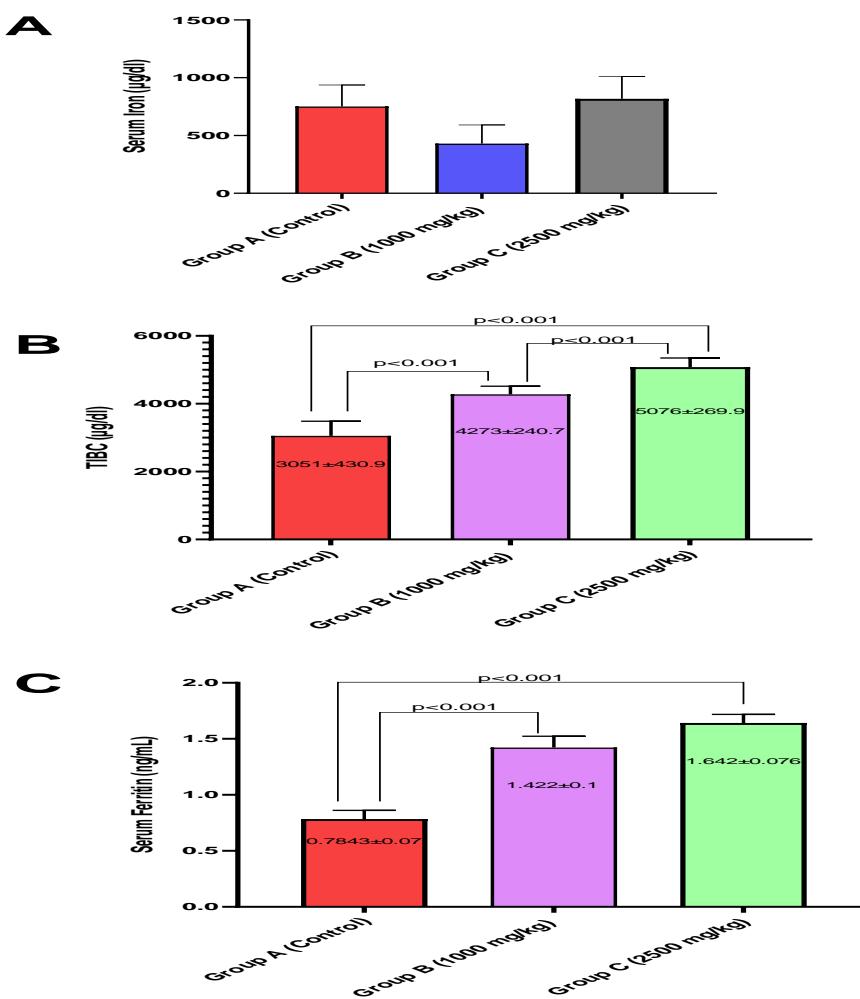


Figure 3: Panel A, B and C shows the effect of soy bean at different concentration on serum iron, TIBC and serum ferritin concentration. . Error bar in all panels showing standard error of mean.

(478.8 \pm 40.50) to control group.

Erythroferrone was significantly higher ($p<0.001$) in group B (696 \pm 23.62) and group C (756.1 \pm 26.89) when compared to control (group A) (657.2 \pm 14.78). There was a significant increase ($p<0.001$) in group C when compared to group B as shown in panel B (Figure 4).

Figure 4 shows the effect of 2 different concentration of *Glycine max* on hepcidin and erythroferrone. Panel A showed that hepcidin was significantly lower ($p=0.007$) in group B (410.2 \pm 53.41) when compared to control (group A) (652.2 \pm 58.85). There was also a significant decrease ($p=0.04$) in hepcidin when comparing group C

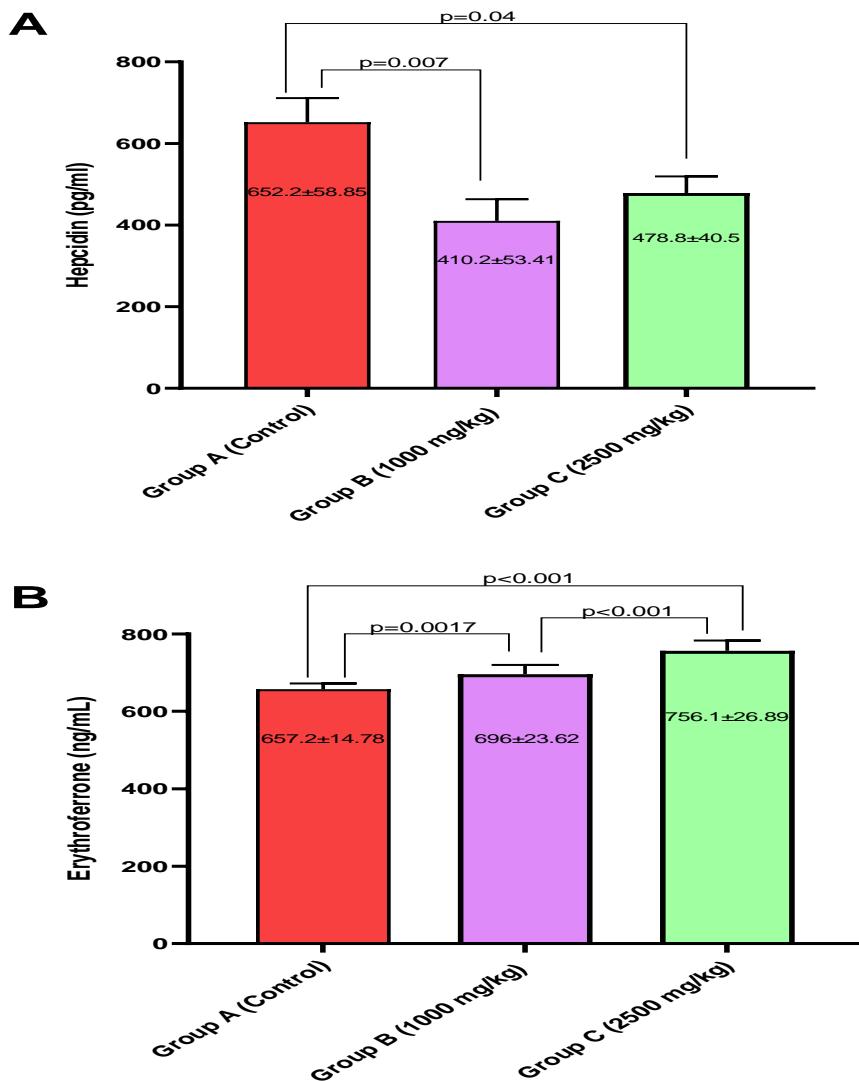


Figure 4: Panel A and B shows the effect of soy bean at different concentrations on hepcidin and erythroferrone concentrations. Error bar in all panels showing standard error of mean.

Table 1 and table 2 shows the effect of aqueous extract of *Glycine max* on complete blood count parameters. The results shows that there was no significant difference ($p>0.05$) in complete blood count parameters when comparing control group and experimental groups.

Table 1: Showing mean \pm SEM of some the haematological indices of female albino wistar rat in the experimental groups compared to the control groups.

Parameters	Control	Test	p value
RBC ($\times 10^{12}/L$)	6.458 \pm 0.1125	6.541 \pm 0.1214	0.667
Haemoglobin (g/dl)	14.86 \pm 0.2880	14.95 \pm 0.2072	0.803
HCT (%)	37.57 \pm 0.5980	37.69 \pm 0.4498	0.876
MCV (fl)	58.3 \pm 1.230	57.85 \pm 0.8433	0.76
MCH (pg)	23.05 \pm 0.5021	22.93 \pm 0.3162	0.835
MCHC (g/dl)	39.51 \pm 0.1906	39.65 \pm 0.1853	0.64
RDW (%)	36 \pm 1.124	36.59 \pm 0.6896	0.642
WBC ($X10^9/L$)	15.29 \pm 2.248	15.28 \pm 0.8544	0.996
Lymphocyte Count (%)	84.02 \pm 2.250	87.47 \pm 0.7887	0.084
Monocyte Count (%)	10.1 \pm 0.7076	9.02 \pm 0.4696	0.204
Granulocyte count (%)	5.88 \pm 1.762	3.51 \pm 0.3874	0.088
Platelet count ($X10^9/L$)	635.3 \pm 33.79	636.8 \pm 22.19	0.971
MPV (μm^3)	6.56 \pm 0.1249	6.515 \pm 0.08592	0.767
PCT (%)	0.4148 \pm 0.02074	0.4127 \pm 0.01183	0.923
PDW (%)	14.59 \pm 0.3622	15.02 \pm 0.2863	0.378

Table 2: Showing mean±SEM of some of the hematological indices of female albino wistar rat of the experimental groups (Group B and C) compared to the control group (Group A).**DISCUSSION**

Parameters	Group A	Group B	Group C	stimulates hepcidin production, resulting in p value
RBC ($\times 10^{12}/L$)	6.458±0.1125	6.532±0.1532	6.549±0.1968	0.911
Haemoglobin (g/dl)	14.86±0.2880	14.98±0.1489	14.92±0.3986	0.96
HCT (%)	37.57±0.5980	37.67±0.3801	37.71±0.8425	0.987
MCV (fl)	57.42±0.9630	57.88±1.129	57.81±1.314	0.957
MCH (pg)	23.05±0.5021	23.01±0.4236	22.85±0.4911	0.951
MCHC (g/dl)	39.51±0.1906	39.76±0.2428	39.54±0.2888	0.734
RDW (%)	36±1.124	35.8±0.7104	37.38±1.168	0.501
WBC ($X10^9/L$)	15.29±2.248	15.14±1.403	15.42±1.053	0.993
Lymphocyte Count (%)	84.02±2.250	88.98±0.7507	85.96±1.247	0.092
Monocyte Count (%)	10.1±0.7076	8.32±0.5318	9.72±0.7345	0.156
Granulocyte count (%)	5.88±1.762	2.83±0.3537	4.19±0.6371	0.165
Platelet count ($X10^9/L$)	635.3±33.79	626.3±26.31	647.2±36.92	0.902
MPV (μm^3)	6.56±0.1249	6.47±0.05783	6.56±0.1655	0.841
PCT (%)	0.4148±0.02074	0.404±0.01415	0.4213±0.01935	0.798
PDW (%)	14.59±0.3622	14.52±0.3441	15.52±0.4149	0.128

Soybean (*Glycine max*) is a widely, inexpensive, and nutritional source of dietary protein (15). Soybeans are among food rich in total phytoestrogens which are present in the form of isoflavones, daidzein, and genistein (3). Phytoestrogens are chemicals that occur naturally in plants and exhibit estrogenic activity. The ones found in soy products are predominantly genistein and daidzein (16). This objective of this study was to investigate the effect of aqueous extract *Glycine max* on the serum hepcidin level, erythroferrone, serum ferritin, serum iron concentration, total iron binding capacity and some haematological parameters. Hepcidin is the main regulator of plasma iron concentrations. Excess iron

increased concentrations of the hormone hepcidin which blocks dietary iron absorption thus preventing further iron loading. When iron is deficient, hepatocytes produce less or no serum hepcidin, allowing more iron to enter plasma (17). In this study, aqueous extract of *Glycine max* significant decreased serum hepcidin levels and significant increased total iron binding capacity (TIBC) levels in the experimental group against the control group. The significant decrease in hepcidin is probably due the presence of estrogen response element present in the hepcidin gene as reported by Ikeda et al. (18). Phytoestrogen have been reported as endocrine disruptors therefore, in the presences of phytoestrogen, normal estrogen could be less functional

because they competitively bind estrogen receptors (19). Hepcidin gene respond to phytoestrogens present in aqueous extract of Soybean, thereby decreasing hepcidin expression. This supports the work of Hou *et al.* (20). They demonstrated the presence of an estrogen response element (ERE) in the promoter region of the hepcidin gene which reduced hepcidin expression thereby increasing iron absorption and elevating total iron binding capacity. The increased iron absorption is probably the reason for the increased levels of serum ferritin observed in this study as reported by Galettia *et al.* (21). In addition, Ikeda *et al.* (18) further reported that a higher hepcidin levels in OVX mice, when estrogen was induced the hepcidin levels dropped, this further hypothesize that role of estrogen in iron metabolism. Furthermore, the level of hepcidin reported in this could also be due to the action of erythroferrone. Our data showed that serum ferritin was significantly higher in the experimental groups. Similarly, Lonnerdal *et al.* (24) reported that when nonpregnant women were given soybean, there was an increase in ferritin. Soybeans have been reported to contain large amount of iron in the form of ferritin. These plant ferritins, which are similar to animal ferritins, are large proteins with molecular weights of about 450,000 and consist of smaller subunits with molecular weights of about 28,000 (25). In this study there was a significant increase in erythroferrone in the experimental groups as compared to controls. It has also been reported that low hepcidin levels favor bone marrow iron supply for hemoglobin synthesis and red blood cells production. Expanded erythropoiesis, as after hemorrhage or erythropoietin treatment, blocks hepcidin through an acute reduction of transferrin saturation and the release of the erythroblast hormone and hepcidin inhibitor erythroferrone (22). Erythroferrone (ERFE)

is an erythropoiesis-driven regulator of iron homeostasis although its mode of action have not been well understood. However, it has been suggested that erythroferrone mediates the suppression of the iron regulatory hormone hepcidin to increase iron absorption and mobilization of iron from stores (23). Both diferric plasma transferrin and stored iron in hepatocytes can stimulate hepcidin synthesis, by distinct mechanisms. However, erythroferrone is an erythroid regulator of hepcidin by suppressing hepcidin in response to increased erythropoietic demand and this also depends on the level of iron (26). This probably explains the reason for the high level of erythroferrone in this study. And this increase in erythroferrone could also suggest the low hepcidin level observed in this study. Our current study also showed that level of erythroferrone and ferritin were higher in the test groups that were administered a higher concentration of aqueous soybean extract, which suggest that its effect is in a dose dependent manner and this is in agreement with previous study that reported that iron status after consumption of soybean for six months, the concentration of hemoglobin and plasma ferritin of participants in soybean group were significantly higher than those of the control as concentration of soybean increases (25). In this study, aqueous extract of *Glycine max* had no significant difference against the control group for red blood cell count, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin concentration, red cell distribution width, white blood cell, lymphocyte count, monocyte count, granulocyte count, platelet count, mean platelet volume, plateletcrit, platelet distribution width. Similar results were also reported by Soung *et al.* (27). Furthermore, Cheng *et al.* (28) also reported no significant changes in haematological parameters in women taking high dose phytoestrogen when

compared to those that were on placebo. Although, other studies have reported that raised levels of serum transferrin saturation (TS), serum ferritin (SF) and serum iron are frequently associated with increased values of haemoglobin and erythrocyte indices (29, 30). The probable reason for our current results in the haematological parameters could be due to the period of administration of the extract. Maybe a longer period of administration could affect the erythrocyte indices.

In conclusion, the results obtained shows that aqueous extract of soybean decreases serum hepcidin and increases total iron binding capacity, serum erythroferrone levels and ferritin concentration. There was no significant differences in red blood cell count, haemoglobin, hematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration and red cell distribution width total WBC, lymphocyte count, platelets count and granulocyte count in all the groups observed.

During this study, some limitation were observed. The age of the female rats could not be ascertained and this have interfered with our results. Another, could also be the interplay between phytoestrogens and phytoferritin which is present in soybeans. So, we recommend that in other studies of this nature, these inferring variables should be well assessed before the start of the study.

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