Investigating the effect of using chitosan nanoparticles containing garlic in improvement of blood function in anemic male rats

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Received: April 2023 Accepted: June 2023

Abstract

This study was aimed at the possibility of using garlic (extract and CNP-G) in alleviating anemia induced by phenylhydrazine, in adult male rats.

Materials and methods: The chitosan nanoparticles containing garlic were prepared in the laboratory. Anemia was induced by phenylhydrazine intraperitoneal (20 mg/kg) for 2 consecutive days. A total of 60adult male albino rats were used in the present study, at the age of 2 months with body weight195±15gm were divided randomly to six equal groups (10 rats for each) and treated as follows for 8 weeks: CG: This group animals left without any treatment like negative control.T1: animals in this group was induced anemia and untreated as a positive group. T2: animals in this group are still normal but treated with a daily dose of 35.4mg/kg of extract garlic given orally by stomach tube. T3: animals in this group were induced anemia and treated with a daily dose of 35.4mg/kg of extract garlic given orally by stomach tube. T4: animals in this group still normal (no anemic) but treated with a daily dose of 35.4mg/kg of CNP-G given orally by stomach tube. T5: animals in this group were induced anemia and treated with daily dose 35.4mg/kg of chitosan nanoparticles containing garlic given orally by stomach tube. At the end of the experiment, all animals were sacrificed and blood samples(5ml) were collected directly from the heart by the cardiac puncture.

Results: Induction of anemia significantly ($p<0.05$) decreased PCV, hemoglobin concentration (Hb), and red blood cell count (RBC), while mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) significantly increased of anemic not treated rats compared to normal control rats. The total protein and albumin showed significantly decreased in anemic group but fibrinogen recorded a significant increase in anemic group as compared with control group. When rats received garlic extract and CNP-G reported increase in the blood electrolytes potassium and iron showed significantly increase in anemic group as compared with the control group. While, sodium and calcium recorded significantly decreased. After treatment by extracting and chitosan nanoparticles containing garlic the K+ and Fe+ increase in all treatment groups and Na+, Ca++ increased in all treatment groups. The result of hormone erythropoietin showed an increase in anemic group and decrease in treatment groups, while VB12 reported decrease in anemic group and increae in treatment groups. While, sodium and calcium recorded significant decreased. After treatment with extract and garlic loaded chitosan nanoparticles the K+ and Fe+ decreased in all treatment groups and Na+ , Ca++ increased in all treatment groups .

Conclusion: This study suggests may be use of CNP-G as antianemic and ability to prevent hemolysis best from garlic extract.

Keywords: CNP-G, Anemia, EPO, VB12, Rat

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Introduction

Anemia, a common public health problem, is characterized as decrease in erythrocyte mass or hemoglobin concentration in the blood leading to reduction in its oxygen carrying capacity **(**Hoffbrand *et al.,* 2006). Morethan two billion people around the world suffer from anemia (Hashim *et al*., 2014). Anemia is more common health problem in the developing countries (Tolentino and Friedman, 2007). Dietary changes and iron supplementation are commonly preferred for the management of anemia. Oral iron therapy has many disadvantages such as insufficient absorption and lack of compliance (Maladkar *et al.,* 2020). Furthermore, consumption of high quantity of these iron supplements can lead to serious health-related complications such as some neurogenic disorders or cancer **(**Saha *et al.,* 2008). All these facts demonstrate the need to have safe and effective alternative for the management of anemia.

Medicinal plants have been a source to control many diseases and anemia is no exception. In traditional systems of medicine including Ayurveda, many plants are claimed to be useful for anemia (Aduwamai and Abimbola, 2018). Previous studies reported antianemic potentials of several Indian medicinal plants (Patil and Navghare, 2019)**.** Few polyherbal formulations are reported to be effective for the treatment of anemia (Aslam *et al.,*2016). These herbal based formulations are preferred by the community as they are cost-effective and have less side effects.

Garlic (*Allium Sativum L.*) is a member of the Alliaceae family, is one of the best essential vegetables all over the world. The importance of garlic is due to its use only for culinary but also for therapeutic and medicinal resolves in both traditional and modern medicine (Singh and Singh, 2008).

Materials and methods

Animals

Sixty albino male rats (*Ruttus* norvegicus) were supplied by the animal house of (University of Babylon - College of Science). Their ages at the start of experiments were 8 weeks, and their weight was 195±15 grams. The rats have been kept under suitable environmental situation. The rats have been housed in cages made up from plastic with dimension 12×15×29 cm. and had free excess to food (standard pellets) and water (ad libitum). The ground sawdust of cages has been changed every week. For adaptation, the rats were remained in animal house about 2 weeks before beginning the experiment.

Induction of anemia

An intraperitoneal injection of 20 mg/kg phenyl hydrazine was applied for two consecutive days to develop hemolytic anemia on the 4^{th} day after the 1^{st} injection in 30 male albino rats (Gheith and El-Mahmoudy, 2018).

Design of experiment

NC Group: animals in this group still normal without any treatment as negative control.

Group T1: animals in this group were induced anemia and treated with distilled water as positive group.
Group T2: animals in this group were treated daily with 34.5 mg/kg of extract garlic given orally by stomach tube.
Group T3: animals in this group were induced anemia and treated daily with 34.5 mg/kg of extract garlic given orally by stomach tube.
Group T4: animals in this group were treated daily with 34.5 mg/kg of CNP-G given orally by stomach tube.
Group T5: animals in this group were induced anemia and treated daily with 34.5 mg/kg of CNP-G given orally by stomach tube.

Sample collection

At the end of the experiment, all animals were sacrificed and blood samples(5 mL) were collected directly from the heart by the cardiac puncture. One ml was put into Ethylenediaminetetraacetic acid (EDTA) tubes for complete blood count measured, another one ml was put into tube with sodium citrate for obtained plasma which was used for fibrinogen biomarker while the remaining 3 ml pushed slowly into disposable tubes containing separating gel and allowed to clot at room temperature for 30 minutes and then centrifuged at 3000×g for approximately 3 minutes. Then the sera were obtained stored at (-20ºC) until physio-biochemical analyses carried out which includes total protein, albumin, and electrolytes.

Hematological studies

All of hematological profile (CBC) have been done by use of an automated auto-analyzer (Horiba A) Biomerieux. In this test, the blood is placed in the vibrator, after which the power switch is pressed. Blood 20 µL is taken by probe, and taken out of the device, after a minute the result was appeared.

Biochemical measurement

Determination of total protein, albumin, and electrolytes

All of which determined fully automated chemistry analyzer Genotech (USA) – SMART-150**.**

Determine plasma Fibrinogen

1- 10 µL serum+190 µL buffer in test tube (Hitach cup)
2- 150 µL of above solution was added to cuvette
3- Added of one magnetic bead into the cuvette
4- Added of 50 µL of liquid fibrinogen reagent into the cuvette
5- Measurement was done using Mindray Semi Automated Coagulation Analyzer

Procedure of erythropoietin (EPO) and VB_{12}

The kits were used in this assay include Rat erythropoietin (EPO), and Rat vitamin B_{12} (VB_{12}), with cat. No. (E0293Ra and E0610Ra,) respectively, using ELISA kit by method of Sandwich-ELISA were determined according to (Jordan, 2005). as following**:**

1. All reagents, standards solution and Samples were prepared as instructed. All reagents were boring to room temperature before use. The assay is performed at room temperature.
2. The number of strips was determined required for the assay, and inserted the strips in the frames for use. The unused strips should be stored at 2-8ºC.
3. Fifty µl of each EPO and VB_{12} standards was added to standard wells. Note: antibody don't add to standard well because the standard solution contains biotinylated antibody.
4. Forty µl sample was added to sample wells and then added 10µL anti-VB_{12} antibody to sample wells, then added 50µL streptavidin-HRP to sample wells and standard wells (Not blank control well). Then, mixed well and covered the plate with sealer and incubated 60 min. at 37ºC.
5. The sealer was removed and washed the plate 5 times with wash buffer. Wells were soaked with at least 0.35 mL wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirated all wells and washed 5 times with wash buffer, overfilling wells with wash buffer. Blotted the plate onto paper towels or other absorbent material.
6. Fifty µl of each EPO and VB_{12} substrate solution A and 50µl of substrate solution B added to each well. Incubated plate covered with a new sealer for 10 minutes at 37°C in the dark.
7. Fifty µl of each EPO and VB_{12} Stop solution was add to all wells. The blue color will change into yellow immediately.
8. The optical density (O.D.) was determined of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Calculations of concentration

A standards curve was plotted for the absorbance versus the concentration of the standards. To calculate each sample concentration of erythropoitein and VB_{12}, first the absorbance value was entered on the y-axis and extended a horizontal line to the standard curve, the point of intersection was found, a vertical line was extended to the x-axis and the corresponding samples concentrations were read to each EPO and VB_{12}.

Statistical analysis

The experiment data was analyzed for statistical by one-way analysis of variance and post hoc comparison using SPSS version 25. All data was reported as mean± SE and statistical significance was accepted at $p \leq 0.05$.

Results

Red blood corpuscular parameters (RBC count, Hb, PCV)

The present study of RBC_s data in (Table 1) showed a significant increase in (T2, T4 , and T5) and there is no a significant ($p \leq 0.05$) difference between them, but still a significant when compared with T1 and negative control group. On the

other hand, blood sample of RBCs showed prominent reduction in T1 group that received PHZ when compared with control and among treated group. In addition to the our data showed a significant decrease in Hb and PCV to recorded mean value (9.92±0.04 and 25.6±0.38) respectively at $p\leq 0.05$, on the other aspect the data of Hb and PCV appear a significant increase to recorded mean value of Hb (13.1±0.6, 12.9±0.17, 13.67±0.21, and 13.8±0.091) respectively, and mean value of PCV (38.01±1.3, 36.1±0.58, 41.08±0.59, and 41.15±0.39), respectively.

Table 1: Effects of garlic nanoparticles on Erythrocytes parameters of anemic male rats.

Groups of experiment	RBCs×10^6	Hb	PCV
NC	6.05 ± 0.27 D	12.06 ± 0.60 B	41 ± 0.39 A
T1	3.43 ± 0.051 E	9.92 ± 0.40 C	25.6 ± 0.38 C
T2	8.21 ± 0.19 A	13.1 ± 0.60 A	38.01 ± 1.3 B
T3	7.20 ± 0.12 B	12.9 ± 0.17 A	36.1 ± 0.58 B
T4	7.72 ± 0.16 A	13.67 ± 0.21 A	41.08 ± 0.59 A
T5	8.2 ± 0.13 A	13.80 ± 0.091 A	41.15 ± 0.39 A
LSD $p\leq 0.05$	0.48	0.97	2.01

The value represent mean±S E, N=10 for each group, Different capital letters indicated significant ($p\leq0.05$) among groups.NC: normal control, T1: anemia positive control, T2: normal received garlic extract, T3: anemic group received garlic extract, T4: normal group received garlic nanoparticles, and T5: anemic group received garlic nanoparticles.

Red blood corpuscles indices

The mean differences ($p\leq 0.05$) of (MCV, MCH, and MCHC) in anemic group showed a significant increase as compared with NC in (Table 2), while treatment groups (T4 and T5) recorded a significant decrease when compared with anemic group and negative control group.

Biochemical parameters

Electrolytes of blood

Table 3 demonstrated the average values of the serum potassium concentration mmol/L in all groups. The present study confirmed that there was a significant ($p\leq0.05$) increase in all mean values of serum K^+ concentration in T2, T3, T4, and T5 groups as compared with NC, on other hand found a significant increase ($p\leq0.05$) in T1 anemic group as compared with negative control group. Serum sodium and calcium concentration in anemic group recorded a significant ($p\leq0.05$) decrease in mean value (127.4±11.1 and 9.06±0.11) respectively as compared with others groups. While (T2 and T5) groups recorded a significant increase as compared with anemic group. While

iron in T1reveal high significant ($p \leq 0.05$) increase in mean value (207±26.9) as compared with NC group and all treatment groups.

Table 2: The effect of garlic nanoparticles on RBC$_s$ indices of anemic male rats.

Groups of experiment	MCV	MCH	MCHC
NC	53.9 ± 0.40 B	18.8 ± 0.27 B	34.5 ± 0.86 B
T1	56.3 ± 1.48 A	19.4 ± 0.32 A	37.4 ± 1.7 A
T2	53.8 ± 0.65 B	18 ± 0.31 B	35.6 ± 0.82 B
T3	55.1 ± 0.42 A	18.16 ± 0.31 B	36.2 ± 0.49 B
T4	53.7 ± 0.73 B	17.9 ± 0.28 BC	32.9 ± 0.38 C
T5	51.4 ± 0.7 C	17.4 ± 0.29 C	32.4 ± 0.39 C
LSD P≤ 0.05	2.32	1.47	2.55

The value represent mean± SE, N=10 for each group. Different capital letters indicated significant ($p \leq 0.05$) among groups.NC: normal control, T1: anemia positive control, T2: normal received garlic extract, T3: anemic group received garlic extract, T4: normal group received garlic nanoparticles, and T5: anemic group received garlic nanoparticles.

Table 3: The effect of garlic nanoparticles on blood electrolytes of anemic male rats.

Groups of experiment	Potassium	Sodium	Calcium	Iron
NC	7.36 ± 0.57 B	138.5 ± 9.2 C	10.6 ± 0.16 C	164 ± 8.01 C
T1	9.98 ± 0.45 A	127.4 ± 11.1 D	9.06 ± 0.11 D	207.7 ± 26.9 A
T2	8.21 ± 0.062 AB	145.4 ± 11.6 A	11.69 ± 0.17 A	166.3 ± 10.5 C
T3	8.58 ± 0.20 AB	143.7 ± 12.5 B	11.21 ± 0.11 AB	169.5 ± 3.2 C
T4	6.64 ± 0.10 B	141.9 ± 12.5 B	11.15 ± 0.17 B	170.9 ± 12.7 C
T5	9.45 ± 0.22 A	147.4 ± 14.7 A	11.4 ± 0.11 A	163.3 ± 22.8 C
LSD P ≤ 0.05	0.93	3.43	0.39	45.4

The value represent mean ± SE. N=10 for each group. Different capital letters indicated significant ($p \leq 0.05$) among groups.NC: normal control, T1: anemia positive control, T2: normal received garlic extract, T3: anemic group received garlic extract, T4: normal group received garlic nanoparticles, and T5: anemic group received garlic nanoparticles.

Total proteins, albumin, and fibrinogen

Data in Table 4 confirmed that serum total protein concentration in the all-treatment groups showed clear significant elevation ($p \leq 0.05$) as compared with anemic group. Although the (T3, T4, and T5) recorded no

significant ($p \leq 0.05$) variation between them. Albumin (g/L) concentration in group that injection by PHZ recorded highly significant ($p \leq 0.05$) reduction when compared with control and all others treated groups. On other hands (T3 and T4) showed no significant ($p \leq 0.05$) difference between them. In addition, the mean difference of fibrinogen showed a significant ($p \leq 0.05$) increase in anemic group as compared with negative control group and decrease in all treatment groups when compared with positive control group.

Table 4: The effect of CNP-G on total protein, albumin, and fibrinogen of anemic male rats.

Groups of experiment	Total protein	Albumin	Fibrinogen
CG	5.95 ± 0.13 C	4.04 ± 0.095 B	269 ± 10.12 B
T1	5.14 ± 0.17 D	3.69 ± 0.13 C	329.4 ± 7.70 A
T2	7.26 ± 0.37 A	4.45 ± 0.15 A	245.7 ± 18.7 B
T3	6.97 ± 0.16 AB	4.25 ± 0.07 AB	239.4 ± 17.6 B
T4	6.37 ± 0.14 B	4.21 ± 0.12 AB	289 ± 9.4 B
T5	6.92 ± 0.32 AB	4.50 ± 0.15 A	244.3 ± 12.1 B
LSD $p \leq 0.05$	0.60	0.35	53

The value represent mean±SE. N=10 for each group. Different capital letters indicated significant ($p \leq 0.05$) among groups.NC: normal control, T1: anemia positive control, T2: normal received garlic extract, T3: anemic group received garlic extract, T4: normal group received garlic nanoparticles, and T5: anemic group received garlic nanoparticles.

Erythropoietin (EPO)

Our data in Figure 1 confirmed high serum concentration of EPO in positive control group that received PHZ at dose 20 mg/kg in mean value (170.9±3.9) while all others treatment groups recorded no significant ($p \leq 0.05$) variation as compared with negative control group and between them.

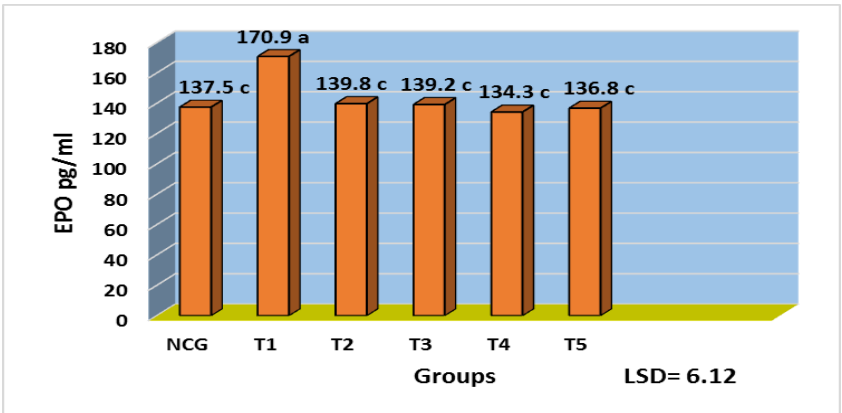

Figure 1: Effect of CNP-G on erythropoietin. The value represent mean±SE; N=10 for each group; Different small letters indicated significant ($p \leq 0.05$) among groups. NC: normal control, T1: anemia positive control, T2: normal received garlic extract, T3: anemic group received garlic extract, T4: normal group received garlic nanoparticles, and T5: anemic group received garlic nanoparticles.

Vitamin B_{12} (VB_{12})

Our data in Figure 2 confirmed high VB12 in serum of treated group that received CNP-G in mean value (0.77±0.031) as compared with anemic group, while T2, T3, and T4 reported no significant (p≤0.05) different as compared with negative control group.

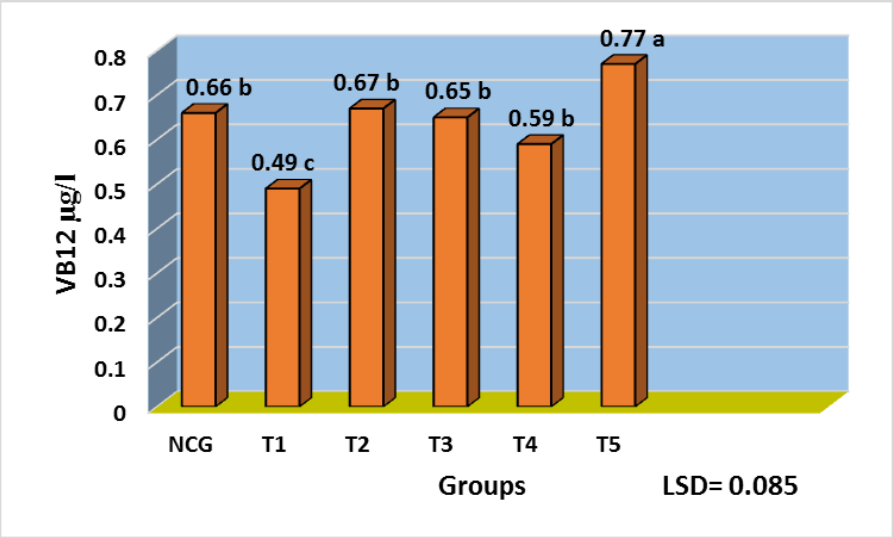

Figure 3.: Effect of CNP-G on vitamin B12 (VB12). The value represent mean± SE; N=10 for each group; Different small letters indicated significant (p≤0.05) among groups. NC: normal control, T1: anemia positive control, T2: normal received garlic extract, T3: anemic group received garlic extract, T4: normal group received CNP-G, and T5: anemic group received CNP-G.

Dissection

Blood parameters

Red blood corpuscular parameters (RBC count, Hb, PCV)

Results of the current study show a direct effect of Phenylhydrazine in creating anemia, wherein a decrease in hemoglobin levels, hematocrite, and the number of red blood cells are found in T1. It has been demonstrated in several studies, that there is a significant association of diagnostic values between RCB, Hb, PCV and blood indices in both humans and rats when exposed to PHZ.

In a study conducted by Igwe *et al*. (2020) for the purpose of inducing anemia in rats by using PHZ, it was found that PHZ leads to a decrease in the levels of hemoglobin and red blood cells witha significant increase (p≤ 0.05) in the level of white blood cells compared to the control group, the reason was attributed to the fact that PHZ alters iron metabolism, interfering with the binding of Erythropoietin receptors, forming Heinz bodies in red blood cells (Vagdatli*et al*., 2010).

Our results of studies concerning the group of animals that received PHZ and the effects on hematological variables represented by the RBC count, PCV, and the Hb, which is the major variable indicator for anemia agreement with the studies of Beshel *et al*. (2018), the results of these studies indicated that the values of these investigated variables are decreased due to the toxicity of PHZ caused by oxidative stress represented

by the generation of free radicals that attack biomolecules that cause damage to the biological system (Sharma and Haldar, 2009).

Giving doses (34.5 mg/kg) of garlic extract and garlic nanoparticles to rats, as shown in Table 1, leads to a significant increase in Hb concentration, PCV, as well as RBC_S compared to the group received PHZ, this is attributed to the role the garlic that play in inhibiting the active radicals produced by PHZ by providing the animals with the natural antioxidants possessed by the plant suchas polyphenolic compounds and antioxidant activity. These compounds are antioxidants that get rid of free radicals and improve normal blood cells production (Reshi*et al*., 2017).

The present study agreement with Suha (2014) who reported that there was increased in Hb, PCV, and RBC_S when treatment groups with garlic The increase in Hb concentration, PCV, and RBC_S count at garlic powder and garlic nanoparticles groups compared with anemic group may be possible related to the end product of garlic metabolism in the body that stimulates the kidney directly to cause formation and secretion of erythropoietin (a potent stimulator of the bone marrow) (Shalaby *et al*., 2006).

Red blood corpuscles indices

Red blood indices (MCV, MCH, and MCHC) are particularly important for the diagnosis of anemia in most animals (Suha, 2014). In the present study the induction with phenyl hydrazine (PHZ) caused increased values of MCV, MCH, and MCHC, as observed in Table 2 and highly decrease in treatment groups (T4 and T5) that received garlic extract and NPs at dose 34.5 mg/kg Erythrocytes that have a normal size or volume (normal MCV) are called normocytic, whereas high and low mean values indicate macrocytic and microcytic respectively. Erythrocytes with normal of hemoglobin concentration (MCHC) are normochromic**,** whereas, abnormally high and low mean values indicate hyperchromic and hypochromic conditions, respectively, though there is no hyperchromic condition (Jordan, 2005) these results are partially agreement with our current study. So, the MCV, MCH and MCHC values in this work were normal suggesting macrocytic hyperchromic anemic condition.

Theseresult contrasted with those of (Tchogou *et al*., 2016) which obtained RBC indices decrease after phenyl hydrazine injection but they were in agreement with (Ponmozhi and Ramya, 2015). The effect of garlic extract and garlic nanoparticles on RBC indices showed a significant decrease. So, it is assumed that the decrease or increase of blood indices may be attributed to a defense reaction against *Allium sativum*, which occurs by stimulation of erythropoiesis (Zare *et al*., 2021).

Biochemical parameters

Electrolytes of blood

In this study, we observed that serum sodium levels were significantly lower ($p \leq 0.05$) and serum potassium levels ($p \leq 0.05$) were significantly higher in anemic group when compared with

negative control group. This result agreement with (Rajagopal *etal*., 2018). In our study, the serum sodium levels were significantly lower and serum potassium levels significantly higher in anemic group which is accordance with results of (Shraf *et al.*,2017). Previous studies have stated that normal red cells have high level of intracellular potassium and low level of sodium within the extracellular environment. On the other hand, the level of potassium is low in the extracellular environment while that of sodium is high. Na^+ and K^+ ions are restricted to their compartment but can penetrate the cellular membrane through Na^+ K^+ ATPase pumps. The red cell Na^+ K^+ ATPase is a ubiquitous enzyme and plays a central role in the regulation of intra- and extra-cellular cationic homeostasis (Nnodim*etal*., 2014).

This study reveals that pooled mean sodium levels in anemia patients had lower than control patients, which was due to dehydration which triggered by movement of sodium into the sickle cell and potassium pooled mean level was reported higher in sickle cell patients as compare to control The possible mechanism was that sickle cell patients usually encountered Cell dehydration and hypoxia, which leads to the loss of potassium from the cell into the extracellular fluid (Antwi-Boasiako *et al.,* 2019). These were in confirmation with studies done by (Madhuri *et al.*, 2019).

The results obtained after treatment with extract and nanoparticles showed increase in both Na^+ and K^+ especially in T5when compared with anemic group. The serum levels of sodium and potassium also increased significantly with higher doses of garlic. This finding is suggestive of a mild hyperkalaemic and hypernatriemic effects. The drug in addition to its other actions may favor an improvement in renal function by increasing sodium and potassium reabsorption. The relieve of hypertension by allicin component of garlic may partly be explained by its secondary effect on possible increase in renal blood flow which enhances renal reabsorption of basic electrolytes like sodium and potassium (Oluwole, 2010) that agreement with present study.

This result is to some extent against the study conducted by Safdar *et al*., (2016) who indicated that garlic had no significant effect on the serum level of sodium in broiler chicks because the chicks were bred in an open shed in the extreme hot and dry climate of DIKhan district, so it is possible that probably due to heat stress endured by the broiler chicks the sera level of sodium were not significantly different for any of the herbal extract.

After treatment with garlic extract and nanoparticles the concentration of calcium increase in all treatment groups especially in T5 as compared with anemic group and negative control group this may be because the garlic contain calcium this results agree with Safdar *et al*., (2016) that showed garlic has increased level of calcium in the sera of broilers, the aqueous extract of garlic probably enhanced the intestinal absorption of calcium by modulating the

activity of Ca-ATPase enzyme present in the plasma membrane. Also, the present study is to some extent in agreement with the study conducted by Mukherjee *et al*., (2006), which suggested the significant effect of oil extract of garlic by promoting intestinal transference of calcium in rats.

Iron homeostasis must be maintained so that cells have sufficient iron for cell growth, but not excess due to its toxicity (De Domenico *et al.,* 2007), in this study, positive control group that received PHZ showed increased serum iron concentration. These results agree with Zangeneh *et al*., (2019) who mentioned that injection of PHZ into rats induced a hemolytic anemia and sequential changes in iron metabolism tests. The greater quantities of iron released from destroyed red blood cells primarily caused hyperferremia (Saito, 2014). When treatment with garlic extract and NPs the iron concentration decrease due to garlic is one of the well-known plants with remarkable antioxidant properties (Agarwal *et al*., 2007) and inhibitory effects on iron availability. Ma *et al*., (2011) suggested that the bioactive garlic polyphenols inhibit iron absorption in a dose-dependent manner in human intestinal Caco-2 cells. Tuntipopipat *et al*. (2009) confirmed that garlic polyphenolic compounds are able to inhibit iron absorption by forming iron complexes in the intestine, making dietary iron less available for absorption.

Total proteins, albumin, and fibrinogen

Intraperitoneal injection of PHZ generated a significant ($p \leq 0.05$) decrease in total protein and albumin in T1. Our study disagreement with Andongma (2014) who found the total protein and albumin showed no significant change in anemic group that may indicate that the synthetic function of the liver has not been significantly affected.

Igwe *et al*., (2020) recorded that TP and ALB decrease in anemic group as compared with normal control. The liver and kidney biomarkers which were significantly elevated by the PHZ agent as shown in the untreated rats. Studies have shown that intravascular hemolysis in any condition may damage the liver and other vascular organs **(**Onyeabo, *et al.,* 2017) apart from hemolysis induced liver injury. After treatment with extract and nanoparticles of garlic observed increase in total protein and albumin these result agreement with Agarwal *et al.,* (2007) that found the serum total protein and albumin increase in fish after 20 days of feeding with garlic that result may be thought to be associated with stronger innate immune response of fish **(**Ma *et al*., 2011).

Ghiasi *et al*. (2012) who found garlic aqueous extract reduced serum value of albumin and no significant changes in total protein due to that garlic has substances such as allicin and diallyl disulfide which are active components of garlic and all of these result is due to mentioned component. Also Al-Sayed *et al*., (2017) reported that no alteration in the levels of total protein and albumin but increase in total globulins was found

in rats that received garlic at 5% these result come in accordance with Jafari *et al*. (2011) who mentioned that garlic powder increase serum γ-globulin in broiler chicks, immune- stimulant effect of garlic may be due to its component (Corzo-Martinez *et al*., 2007)

Also after treatment fibrinogen level decrease. Our result agreement with Reddy *et al*., (2017) that reported decrease of fibrinogen after administration of garlic. One Indian study showed that intake of garlic in a regular diet could remove fibrin clots could remove fibrin clots and reduce the incidence of cardio vascular disease (Bordia *et al*., 1998). Almost all human researches on fibrinolytic activity of garlic have been found to have positive effect in fibrinolysis (Reddy *et al*., 2017). Garlic reduced fibrinogen level in hyperlipidemic rats after 4 weeks of treatment, compared with control group. This reduction due to garlic has a potent fibrinolytic activity (Alhamami *et al*., 2006).

Erythropoietin (EPO)

The present study reported that EPO increase in anemic group as compared with control and all treatment groups. This is may be due to in severe anemia, the coexisting hypoxia stimulates erythropoiesis through increased kidney synthesis and release of EPO (Kautz *et al*., 2014)**.**This leads to suppression of hepcidin transcription by erythroferrone (ERFE), an EPO target gene produced by erythroblasts (Pagani *et al*., 2019), by molecules (e.g., PDGF-BB) released by other tissues (Sonnweber *et al*., 2014).

When treatment anemic rats with garlic extract and CNP-G observed significant decrease in EPO level this may be due to increase the erythropoiesis rate to production more RBC. Our results supported by Akgul *et al*. (2010) who reported decrease EPO level after treatment with garlic because garlic consumption not only causes increased energy demand from the faster RBC turnover but also increases the production of CO, which in turn stimulates splenic erythropoiesis by an erythropoietin-independent mechanism, thus completing the sequence of feedback regulation for RBC metabolism.

Vitamin B_{12} (VB_{12})

The present study showed significant decrease of VB_{12} in anemic group compare with other groups. Human B_{12} deficiency is caused mainly by lack of animal source food or lack of intrinsic factor (FI). Pathological causes are mainly related to malabsorption due to various reasons, pernicious anemia being the most common amongst them (Ata *et al*., 2020).

After treatments by garlic, the level of VB12 in blood increased this may be because the garlic contains vitamins. Fresh garlic contains micronutrients essential for metabolism and physiological functions in the human body, including multiple vitamins (about 0.058% in GF, such as vitamin C, vitamin E, thiamine (VB1), and riboflamin (VB2)), and various trace elements (about 0.7% in FG such as

calcium, sodium, zinc, germanium, and selenium (Qiu *et al*., 2019).

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