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Effect of garlic nanoparticles on bone marrow function and antioxidants status in experimentally anemic male rats

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Abstract---Background and Objective: This study was aimed the possibility of using garlic (extract and nanoparticles) in alleviating of bone marrow function and antioxidant status in anemic male rats induced by phenylhydrazine(PHZ). Materials and methods: The CNP-G was prepared according to [1]. Anemia was induced by phenylhydrazine intraperitoneal (20 mg/kg) for 2 consecutive days. A total of 60 adult male albino rats were used in the present study, at the age of 2 months with body weight 195 ± 15 gm were divided randomly to six equal groups (10 rats for each) and treated as follows for 8 weeks:- Control group:- in this group animals left without any treatment like negative control.T1: animals in this group was induced anemia and untreated as positive group. T2: animals in this group still normal (no anemic) but treated with daily dose 35.4mg/kg of extract garlic given orally by stomach tube. T3: animals in this group were induced anemia and treated with daily dose 35.4mg/kg of extract garlic given orally by stomach tube. T4: animals in this group still normal (no anemic) but treated with daily dose 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 CNP-G 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 in rats significantly ($P<0.05$) decreased red blood cell count (RBC), hemoglobin concentration (Hb), PCV, and total antioxidant capacity . The affect anemic on bone marrow showed by increase adipose tissue within bone marrow. After treatment rats with garlic

extract and CNP-G recorded significantly increase in RBC count, Hb, PCV, and MDA concentration. While the garlic extract and CNP-G decrease adipose tissue. Conclusion: This study suggests may be use of CNP-G as antianemic and ability to prevent hemolysis best from garlic extract. Confirmation of the studied indicator of blood that gave the best results as evidence of treatment.

Keywords--CNP-G, anemia, bone marrow anatomy, total antioxidant capacity, malondialdehyde.

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 [2]. More than two billion people around the world suffer from anemia [3]. Anemia is more common health problem in the developing countries [4]. 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 [5]. Furthermore, consumption of high quantity of these iron supplements can lead to serious health-related complications such as some neurogenic disorders or cancer [6]. 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 [7]. Previous studies reported antianemic potentials of several Indian medicinal plants [8]. Few polyherbal formulations are reported to be effective for the treatment of anemia [9]. 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 [10]. Garlic contains a higher concentration of sulfur compounds (such as allicin, diallyl disulphide, S-allylcysteine, and diallyl trisulfide) which are responsible for its various kinds of medicinal effects. It is eaten up either as raw vegetable (fresh leaves or dried cloves), or after processing in the form of garlic oil, garlic extracts, and garlic powder with changes in chemical composition and bioactive compounds content between the various forms [11].

Materials and methods

Animals

Sixty albino male rats were supplied by the animal house of College Science/ University of Babylon. 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 x 15 x 29 cm and had free excess to food (standard pellets) and water (ad libitum). The ground sawdust of cages have 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 phenylhydrazine was applied for two consecutive days to develop hemolytic anemia on the 4th day after the 1st injection in 30 male albino rats.[12]

Design of experiment

- **NC Group:** animals in this group still normal without any treatment as negative control.
- **Group T1 :** animals in this group was induced anemia and treated with distilled water as positive group.
- **Group T2 :** animals in this group was treated daily with 34.5 mg/kg of extract garlic given orally by stomach tube.
- **Group T3 :** animals in this group was induced anemia and treated daily with 34.5 mg/kg of extract garlic given orally by stomach tube.
- **Group T4 :** animals in this group was treated daily with 34.5 mg/kg of garlic loaded CSNPs given orally by stomach tube.
- **Group T5 :** animals in this group was induced anemia and treated daily with 34.5 mg/kg of garlic loaded CSNPs given orally by stomach tube.

Sample collection

At the end of the experiment, all animals were sacrificed and blood samples(5ml) were collected directly from the heart by the cardiac puncture . One ml was put into EDTA tubes for CBC 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(-20°C) 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.

Measuring of antioxidant and oxidation status(T-AOC and MDA)

The kits were used in this assay include Rat total antioxidant capacity(T-AOC), and Rat Malondialdehyde (MDA) with Cat. No. (E3901Ra , and E0156 Ra) respectively, using ELISA kit by method of Sandwich-ELISA were determined according to [13].

Histological study

Preparation of 10% EDTA (pH 7.4) solution

Dissolve 100 gm of disodium ethylene diaminetetraacetate (EDTA) in 1000 ml of distilled water using magnate stirrer. NaOH was used to adjust the pH to 7.4.

Preparation of femoral bone sample (Luna, 1968)

Femoral bone samples were collected and fixed in 10% formalin for 48 hours. After fixation, femoral bone samples were decalcified by immersing the bones in 10% EDTA (pH7.4) solution (solution size 10 times greater than sample size) for 1 to 2 months. The EDTA solution was changed every 3 days until the decalcification process finished. Bone samples were checked weekly using fine needle to confirm decalcification process of bone samples finished. After decalcification of bone samples confirmed, decalcified bone samples were sliced to 0.5 cm thick and placed in plastic cassettes for dehydration using an automated tissue processor (Histo-Line ATP700, Italy), before embedded in paraffin using the routine paraffin embedding method using tissue embedding system (HESTION TEC2800-C, China). The bone tissue samples were then trimmed and sectioned at 4µm thickness using semiautomatic microtome (Histo-Line MRS3500, Italy). Then the bone tissue sections were placed carefully in water bath (FALC BI, Italy) and mounted on glass slides using a hot plate (K&K HYSH11, Korea).

Subsequently, the bone tissue sections were deparaffinised by two changes of xylene for 2 minutes each and rehydrated by three changes of different ethanol dilution (100, 90 and 70%) for 2 minutes each, respectively. The tissue sections were then further rinsed in tap water and stained with Harris's haematoxylin for 5-8 minutes and washed in tap water for 2 minute. Subsequently, bone tissue sections were differentiated in 1% acid alcohol solution for 20 seconds to remove the excessive haematoxylin stain and washed in tap water for 1 minute. Tissue sections were then immersed in 0.2% ammonia water solution for bluing haematoxylin stain for 1 minute and wash in running tap water for 5 minutes. Bone tissue sections rinsed in 95% alcohol for 20 seconds and counterstained with eosin stains for 5 minute. Then, tissue sections were dehydrated by three changes of different ethanol dilution (70, 90 and 100%) for 2 minutes each, respectively and cleared by two changes of xylene for 2 minutes for each. Tissue section were observed using a light microscope at 40,100, 200, 400 and 1000x magnifications.

Statistical analysis

The experimental data was analyzed for statistical significant by one-way analysis of variance and post hoc (LSD) comparison using SPSS version 25. All data was reported as mean ± SD and statistical significance was accepted at $P \leq 0.05$ [14].

Results

Hematological studies

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

The present study of RBCs data table (3-1), showed a significant increase in (T2,T4, and T5) and there is no a significant ($P \leq 0.05$) difference between(T2 and T5) but found significant differences between T4 and T5, but still a significant when compared with T1 and negative control group. On the other hand blood samples 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 of anemic group 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 in treatment groups 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(41 ± 0.39 , 25.6 ± 0.38 , 38.01 ± 1.3 , 36.1 ± 0.58 , 41.08 ± 0.59 , and 41.15 ± 0.39) respectively.

Table 3.1

Effects of garlic nanoparticles on Erythrocytes parameters of anemic male rats

Groups of experiment	RBCs $\times 10^6$ /mm ³	Hb g/dl	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 B	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 \pm S E
- 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.

Measuring of antioxidant and oxidation status Total antioxidant capacity (T-AOC)

Our data in figure 3-1 appear a significant ($P \leq 0.05$) increase of total antioxidant capacity in all groups treatment that received garlic extract and garlic nanoparticles, and high increase in T5 that received garlic nanoparticles when compared with anemic group and showed no significant ($P \leq 0.05$) different when compared with negative control.

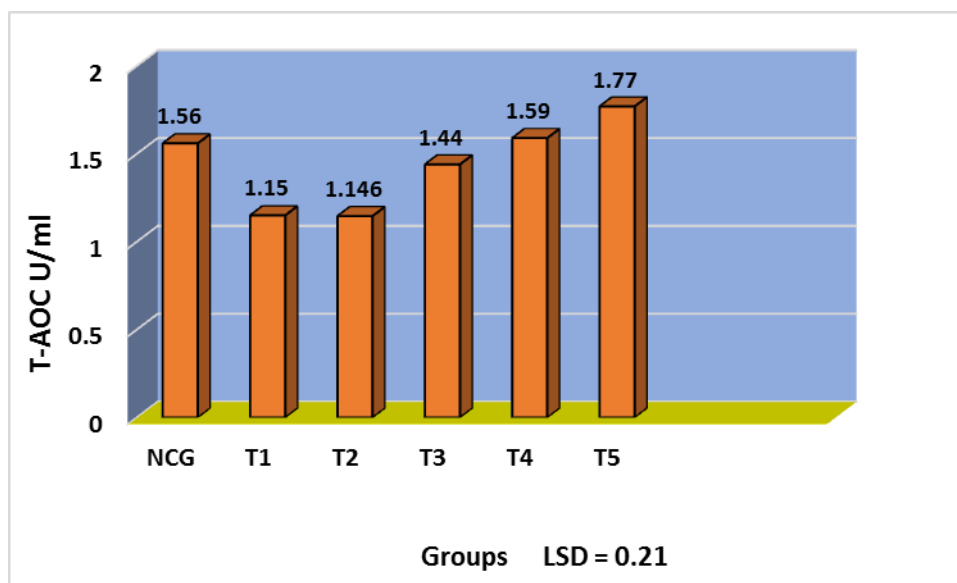


Figure 3.1. Effects of garlic nanoparticles on T-AOC

- The value represent mean \pm 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.

Malondialdehyde (MDA) concentration

The present study illustrated in figure 3-2 showed a significant increase ($P \leq 0.05$) in serum MDA concentration of anemic group of mean value (43.2 ± 0.61) as compared with negative control and all treatment groups that recorded mean value (33.9 ± 0.78 , 32.9 ± 0.54 , 34.2 ± 1.29 , 32.8 ± 0.48 , 30.4 ± 0.58) respectively, and T5 recorded high decreased in mean value of MDA.

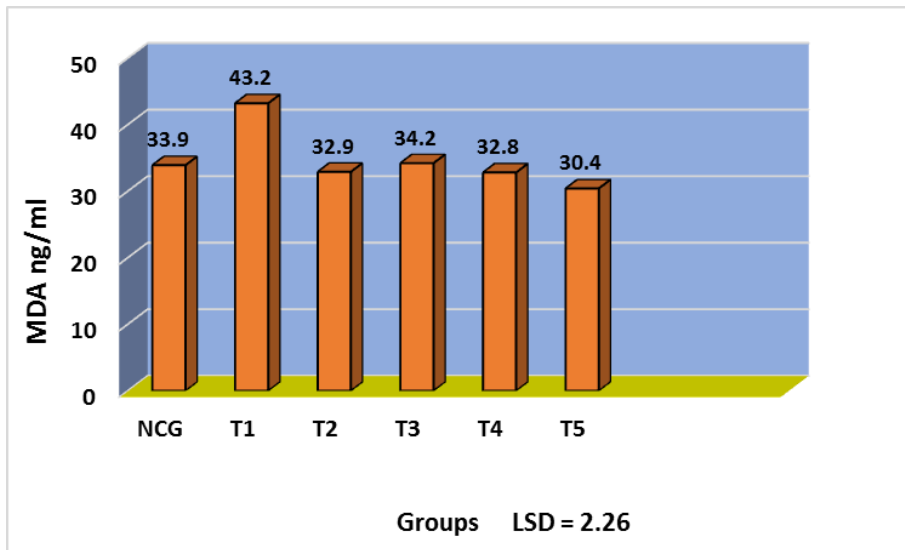


Figure 3.2. Effects of garlic nanoparticles on MDA concentration

- The value represent mean \pm 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.

Histological study

Examination of femur bone marrow of anemia-induced rats figure 3-3, showed the severe hypoplasia of erythroid cells was observed, where the low density of erythroid cells formed huge spaces (arrows) within the bone marrow tissue, also these spaces formed more than 50% of all bone marrow tissue. Where, the severe hypoplasia of erythroid cells indicated failure of bone marrow to produce erythrocytes as compared with negative control group in figure 3-4, that showed the healthy proliferation activity of bone marrow. While when the rats anemic received garlic extract and nanoparticles at does 34.5 mg/kg showed the hypoplasia of erythroid cells was observed, where the decrement in erythroid cells replaced with fatty cells that observed as vacuoles (arrows) within the bone marrow tissue, also the fatty tissue in affected bone marrow tissue formed about 30% of all bone marrow tissue. Where, the hypoplasia of erythroid cells indicated the hypo-proliferative bone marrow.

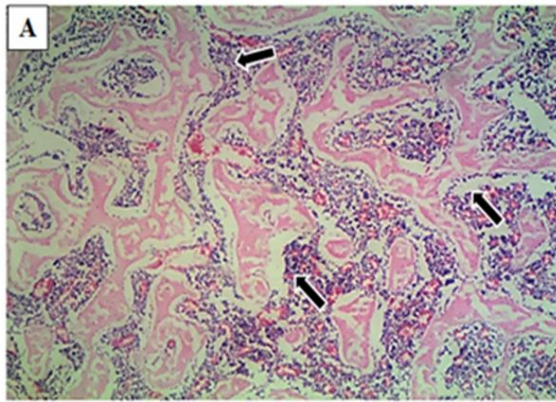


Figure 3.3. Photomicrograph of bone marrow of femur bone of negative control group of rats

The normal architecture of bone marrow was observed, where the bone marrow fill all the space between bone trabeculae tissue (arrows) of the femur bone heads, indicating the healthy proliferation activity of bone marrow. H&E. x40.

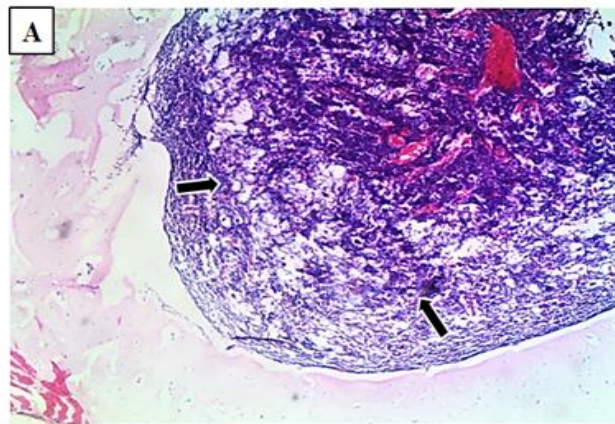


Figure 3.4. photomicrograph of bone marrow of femur bone of T1 group rats (anemia positive control)

The severe hypoplasia of erythroid cells was observed, where the low density of erythroid cells formed huge spaces (arrows) within the bone marrow tissue, also these spaces formed more than 50% of all bone marrow tissue. Where, the severe hypoplasia of erythroid cells indicated failure of bone marrow to produce erythrocytes. H&E. x40.

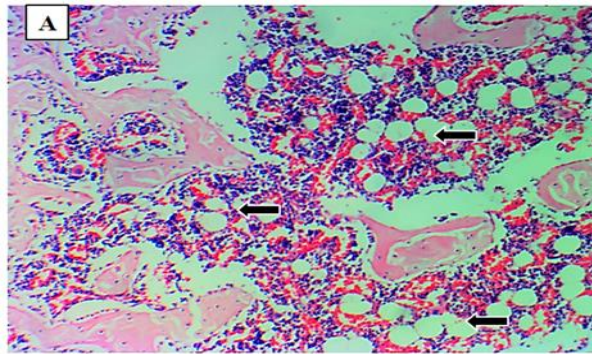


Figure 3.5. Photomicrograph of bone marrow of femur bone of T2 group rats (normal received garlic extract)

The hypoplasia of erythroid cells was observed, where the decrement in erythroid cells replaced with fatty cells that observed as vacuoles (arrows) within the bone marrow tissue, also the fatty tissue in affected bone marrow tissue formed about 30% of all bone marrow tissue. Where, the hypoplasia of erythroid cells indicated the hypo-proliferative bone marrow. H&E. x100.

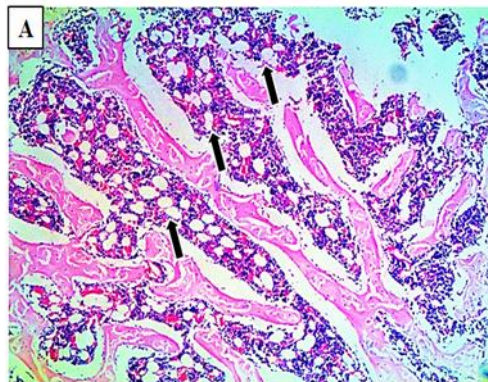


Figure 3.6. photomicrograph of bone marrow of femur bone of T3 group rats (anemic group received garlic extract)

The hypoplasia of erythroid cells was observed, where the decrement in erythroid cells replaced with fatty cells that observed as vacuoles (arrows) within the bone marrow tissue, also the fatty tissue in affected bone marrow tissue formed about 30% of all bone marrow tissue. Where, the hypoplasia of erythroid cells indicated the hypo-proliferative bone marrow. H&E. x40.

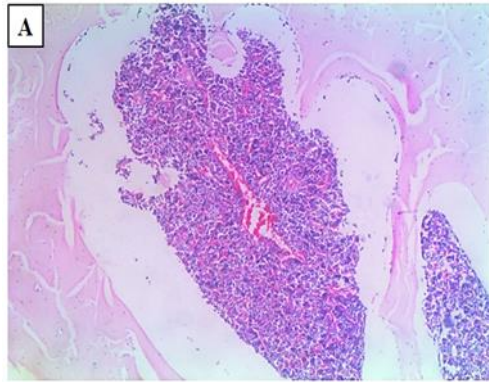


Figure 3.7. photomicrograph of bone marrow of femur bone of T4 group rats(normal group received CNP-G)

The normal architecture of bone marrow was observed, where the bone marrow filled space inside femur bone body indicating the healthy proliferation activity of bone marrow. The mitotic activity (arrows) was also observed with presences of megakaryocytic cells (arrowheads). H&E. x40.

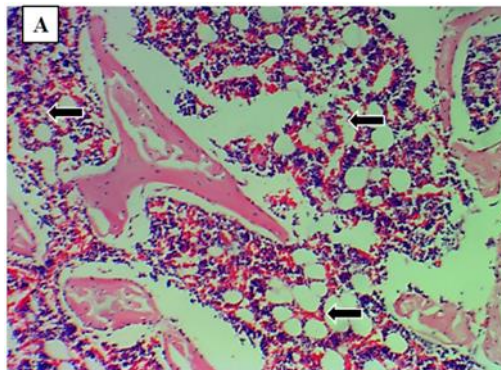


Figure 3.8. Photomicrograph of bone marrow of femur bone of T5 group rats(anemic group received CNP-G)

The hypoplasia of erythroid cells was observed, where the decrement in erythroid cells replaced with fatty cells that observed as vacuoles (arrows) within the bone marrow tissue, also the fatty tissue in affected bone marrow tissue formed about 30% of all bone marrow tissue. Where, the hypoplasia of erythroid cells indicated the hypo-proliferative bone marrow. H&E. x100

Discussion

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 RBC, Hb, PCV and blood indices in both humans and rats when exposed to PHZ. In a study conducted by Igwe *et al.*, [15] 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 with a significant increase ($P \leq 0.05$) 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 [16]. 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.*, [17], 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 [18,19]. Giving doses of garlic extract and garlic loaded CNP-G (34.5 mg/kg) to rats, as shown in table 3-1, leads to a significant increase in Hb concentration, PCV, as well as RBCs compared to the group received PHZ, this attributed to the role the garlic which inhibits the active radicals produced by PHZ by providing the animals with the natural antioxidants possessed by the plant such as polyphenolic compounds and antioxidant activity.

These compounds are antioxidants that get rid of free radicals and improve normal blood cells production [20,21]. The present study agreement with Suha, [22] who reported that there was an increase in Hb, PCV, and RBCs when treatment groups with garlic. The increase in Hb concentration, PCV, and RBCs count at garlic powder and garlic nanoparticles groups compared with anemic group may be possibly 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) [22]. Total antioxidant play an important role in protection alongside damage caused by oxidizing environments, while malondialdehyde (MDA) is a main product of lipid peroxidation as in figure 3-1 and 3-2. The successive formation of PUFA hydroperoxide and β cleavage is the alternative mechanism for MDA generation and the main source of MDA generation *in vivo* [24]. In present study anemic group showed a significant decrease in T-AOC levels with significant increase in MDA comparing to negative control group and treatment groups.

These results agreement with [25]. Ashour, [26] reported that PHZ induced significant decrease in GSH level. As PHZ induced increase in reactive oxygen species (ROS) and lipid peroxidation with decrease glutathione. Our results indicated that the effect of PHZ on serum concentrations of superoxide dismutase and malondialdehyde were found to be essentially contrasting: significant decrease in superoxide dismutase with corresponding significant increase in malondialdehyde concentration [27]. According to Ryan and Prescott [28] when phenolic compounds are subjected to *in vitro* digestions, they are transformed into various structural forms, with different functions and chemical properties. Such functions and properties might provide various results of antioxidant activity which are estimated through various approaches.

Thus, antioxidant capacity measurement through more than one approach was suggested by Akillioglu and Karakaya [29], phyto-nutraceuticals have been utilized since time in-memorial for improving the health of humans. Individuals consuming diet abundant in bioactive components are at low risks of chronic disparities, thus decreasing the rates of morbidity and mortality [30]. Such

phytochemical compounds are rich in antioxidants that have the ability of neutralizing free radicals via donating electrons, thus converting them into certain harmless compounds. Hence, they can assist in preventing or decreasing different physiological threats caused by free radical formation [31]. Comparing to anemic group, treatment groups with garlic extract and nanoparticles showed increase in total antioxidant capacity and decrease in malondialdehyde levels. Some studies [32, 33] referred that when treated with garlic 5% increase T-AOC with decrease in MDA. Garlic extract exhibit antioxidant activities by scavenging the free radicals generated in rat kidney [34]. Also, these result agree with Ghorbel *et al.*, [35] who have demonstrated that garlic, by its antioxidant power, has nephroprotective properties, which have been attributed to the active compound S-allyl cysteine (SAC). According to Hassan *et al.*, [36], garlic oil treatment induced a clear improvement of kidney function, due to its antioxidant properties in scavenging free radicals and reducing levels of lipid peroxidation. SAC is reported to suppress the formation of superoxides, while diallyldisulfide (DADS) and diallylsulfide (DAS) scavenge hydroxyl radical, thus enhance in vivo endogenous antioxidant system and prevent oxidative stress [37].

Histopathological evaluation indicated that anemia-induction by phenylhydrazine administration caused pathological changes of moderate grade in bone marrow tissues the present results agree with [38]. These pathological changes were reversed by garlic extract and NPs treatment and restored histomorphological features of these tissues near to normal. The observed effect may be due to potent antianemic potential of garlic. Different plants and plant extracts can also stimulate differentiation and proliferation of cells in the bone marrow. Garlic extract and NPs improve the cellular in bone marrow may be due to the differences in the utilization of garlic components and the concentration of the active ingredients, that are stimulatory cause an up-regulation of the erythropoietin-receptors on the proliferative bone marrow cells, also, William [39], who found garlic extract is an active oxygen scavenger. It is thus possible that garlic components compete with Hb in the RBC for oxygen resulting in hypoxia, which then stimulates Hb synthesis and RBC production. It is also possible that the end products of garlic metabolism in the body stimulates the kidney directly to cause formation and secretion of erythropoietin (EPO), a potent stimulator of the bone marrow's pluripotent stem cells. It has been reported that PHZ increases formation of reactive oxygen species and thereby causes damage to RBCs. Flavonoids have potent antioxidant property and have capacity to prevent or repair this damage to red cells [40].

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