وزارة التعليم العالي والبحث العلمي

جامعة بابـــــــل

كليـــة العلـوم

قسم علـــــوم الحياة

تأثير الثوم المحمل على الجسيمات النانوية في فقر الدم التحللي المستحث بالفنيل هايدرازين في ذكور الجرذان

أطروحـــــــــــــة

مقدمة الى مجلس كلية العلوم/ جامعة بابل وهي جزء من متطلبات نيل درجة دكتوراه فلسفة في العلوم/ علوم الحياة/علم الحيوان

من قبل

حوراء حامد ناجي غضب

بكـــالوريوس علوم حياة/ جامعة بابل/ 2005

مـــــــــــاجستير علوم حياة / جامعة بابل/ 2016

إشراف

أ.د. رحاب عيدان كاظم **أ.د. حيدر عبدالكاظم الزاملي**

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الخلاصة

أجريت الدراسة الحالية في كلية العلوم بجامعة بابل للفترة من شباط 2021 الى تشرين الاول 2021 على الجرذان المختبرية لدراسة إمكانية استخدام خلاصة الثوم وخلاصة الثوم المحملة على الجسيمات النانوية للكيتوسان في علاج فقر الدم المستحث بمادة الفينيل هيدرازين في ذكور الجرذان البالغة ، تم استيراد خلاصة الثوم (*Allium Sativum*) من بكين (الصين) ، تم تحليل مركبات خلاصة الثوم بواسطة كروماتوكرافيا الغاز- مطياف الكتلة وظهر وجود 20 مركب لمحتوى خلاصة الثوم بنسب مختلفة كان اكثرها ثنائي كبريتيد الاليل (66,7%)، ثنائي كبريتيد ثنائي الاليل (10,01%) و ثنائي كبريتيد، مثيل 2- بروبونيل (8,87%). بعد ذلك عمل على تحميل جزيئات خلاصة الثوم على الجسيمات النانوية للكيتوسان وتم اختبار مواصفاتها بعد التحميل بواسطة اختبار حجم الجسيمات الذي كشف ان حجم جزيئات خلاصة الثوم 11.6nm ، بينما الحجم بعد التحميل 25.9nm. بالنسبة لاختبار جهد زيتا اظهر الشحنة السطحية للكيتوسان ($30.0mV^{+}$)، ثلاثي الفوسفات المتعدد ($19.62mV^{-}$)، خلاصة الثوم ($28.80mV^{+}$)، خلاصة الثوم محملة على الكيتوسان ($48.43mV^{+}$). أظهر أختبار طيف الاشعة تحت الحمراء وجود مجاميع وظيفية مختلفة في المواد هي (OH, NH, HCH, C-O,C=C,C=O, C-N) التي تعمل كعامل اختزال واستقرار. اما TEM فقد اظهرت الجسيمات النانوية للكيتوسان بطبقات ممتزة على السطح تمثل المواد ، حيث ظهرت لخلاصة الثوم جسيمات بسطوح خشنة بأشكال كروية الى غير منتظمة.

وقد اجريت الدراسة بتجربة داخل جسم الجرذان الحي، على(60) ذكرا من الجرذان، تضمنت هذه التجربة على (6) مجاميع للحيوانات (10 جرذان لكل مجموعة، تم تجريعها لمدة 60 يوما)، المجموعة الاولى مجموعة السيطرة الطبيعية التي جرعت فمويا من المحلول الفسيولوجي المتعادل يوميا، T1 مجموعة حقنت من الفينيل هيدرازين 20 ملغم/كغم داخل البريتون، T2 هذه المجموعة جرعت فمويا بجرعة 34.5 ملغم/كغم يوميا، T3حقنت المجموعة 20 ملغم/كغم من الفينيل هيدرازين و 34.5 ملغم/كغم يوميا من خلاصة الثوم فمويا ، T4 هذه المجموعة جرعت فمويا خلاصة الثوم المحملة على الجسيمات النانوية للكيتوسان بجرعة 34.5 ملغم/كغم يوميا، و T5 حقنت ب 20 ملغم/كغم من الفينيل هيدرازين و 34.5 ملغم/كغم من خلاصة الثوم المحملة على الجسيمات النانوية للكيتوسان فمويا. بعدها تم التضحية بالحيوانات تحت التخدير العميق باستخدام الزايلازين- كيتامين وجمع الدم عن طريق ثقب القلب وعزل عظم الفخذ للقسم النسيجي. بينت النتائج ان استحداث فقر الدم في الجرذان احدث انخفاضا

معنويا($P<0.05$) بالمقارنة مع مجموعة السيطرة السالبة في المتغيرات الدموية متمثلة في عدد خلايا الدم الحمر (RBC)، ومستوى الهيموكلوبين (Hb) وحجم الخلايا الدم المرصوص (PCV) وادى الى زيادة في متوسط حجم كرية الدم(MCV)، متوسط الهيموكلوبين (MCH)،متوسط تركيز الهيموكلوبين في الكرية (MCHC) و عدد خلايا الدم البيض (WBC)، كما اظهر معدل ترسيب كريات الدم الحمر والصفائح الدموية زيادة معنوية في مجموعة فقر الدم بالمقارنة مع مجموعة السيطرة. بعد العلاج بخلاصة الثوم وخلاصة الثوم المحملة على الجسيمات النانوية للكيتوسان سجلت ال ESR والصفائح الدموية انخفاضا. كما اظهر البروتين الكلي والالبومين انخفاضا معنويا في مجموعة فقر الدم ولكن الفايبرينوجين سجل زيادة معنوية في هذه المجموعة مقارنة بمجموعة السيطرة. عندما جرعت الجرذان خلاصة الثوم وال CNP-G اظهرت زيادة في الكتروليات الدم، حيث اظهر البوتاسيوم والحديد زيادة معنوية في مجموعة السيطرة الموجبة مقارنة بمجموعة السيطرة السالبة. بينما ايونات الصوديوم والكالسيوم سجلت انخفاض بشكل ملحوظ. بعد العلاج بالخلاصة وال CNP-G ينخفض ال K^+ وال Fe^{++} في جميع مجموعات المعالجة وال Ca^+ وال Na^+ سجلت زيادة في جميع مجموعات المعالجة. كما ادى استحداث فقر الدم في الجرذان الى انخفاض معنوي ($P<0.05$) في مضادات الاكسدة الكلية وزيادة في MDA ، كما سجلت بعد العلاج بخلاصة الثوم وال CNP-G زيادة معنوية في ال TAO-C وانخفاض في ال MDA وسجل هرمون الهيموبكسين انخفاضا معنويا في مجموعة فقر الدم مقارنة بمجموعة السيطرة السلبية، بينما سجلت المجموعات المعالجة زيادة معنوية. اظهر هرمون الاريثروبويتين زيادة معنوية في المجموعة المستحثة بفقر الدم ولكن سجل انخفاضا في المجموعات المعالجة، ارتفاع VB12 في مجموعة T5 التي عولجت ب CNP-G مقارنة بمجموعة فقر الدم، في حين ان T2 و T3 و T4 لم يلاحظ فيها اي اختلاف معنوي. مقارنة مع مجموعة السيطرة السلبية كان هناك ارتفاع معنوي في انزيم ال cyclooxygenase في مجموعة فقر الدم مقارنة مع مجموعة السيطرة وجميع المجموعات العلاجية الاخرى، بينما سجل انخفاضا معنويا في السيروم اميلويد في مجموعة ال T4 وال T5 التي عولجت ب CNP-G مقارنتها بمجموعة فقر الدم ، ومع مجموعة ال T2 وال T3 التي عولجت بخلاصة الثوم. ان تأثير فقر الدم على نخاع العظم ظهر من خلال زيادة الانسجة الدهنية داخل النخاع ، وبعد العلاج بخلاصة الثوم وخلاصة الثوم المحملة على الجسيمات النانوية للكيتوسان سجل انخفاضا كبيرا في الانسجة الدهنية.

يمكن الاستنتاج من الدراسة الحالية ان خلاصة الثوم وخلاصة الثوم المحملة على الجسيمات النانوية للكيتوسان كان لها دور فعال في الحد من فقر الدم. من جانب اخر يمكن الاستنتاج ان التحميل النانوي كان له دور مهم في زيادة فعالية المادة العلاجية (الثوم) للجرذان السليمة والمستحث فيها فقر الدم وفقا لخواص المادة الاساسية.

Republic of Iraq
Ministry of Higher Education
& Scientific Research
University of Babylon-College of Science
Biology Department

Effects of Garlic Loading Nanoparticles on Hemolytic Anemia Induced by Phenylhydrazin in Male Rats

A Thesis

Submitted to the Council of the College of Sciences/ University of Babylon, in Partial Fulfillment of the Requirements for the Degree of Doctoral of Philosophy in Biology/Zoology

By

Hawraa Hamed Naji Gethab

B.Sc. in Biology\University of Babylon \2005

M.Sc. In Biology\ University of Babylon \2016

Supervised by

Prof. Dr.Rihab Edan Kadhim

Prof. Dr. Hayder Abdul Kadem Al-Zamily

2022 A.D **1444 A.H**

Certification

We, certify that this thesis "**Effects of Garlic Nanoparticales in Experimentally Anemic Male Rats** " was prepared under our supervision at the department of Biology, College of Sciences, University of Babylon as a partial fulfillment of the requirement for the degree of Doctorate of physiology in Biology/Zoology.

Supervisor

Prof. Dr. Rihab Edan Kadhim

College of Sciences

University of Babylon

/ / 2022

Supervisor

Prof. Dr. Hayder Abdul Kadem

College of Veterinary Medicine

University of Al-Qadiseya

/ / 2022

In review of the available recommendation, I forward the present thesis for debate by the examining committee.

Assist. Prof. Dr Adi Jasim Abd Al-Rezzaq

Head of Department of Biology

College of Sciences

University of Babylon

/ / 2022

Dedication

To the soul of my

Father and brother…

TO THE TENDER of MY

MOTHER

To my beloved and dear to my heart

my husband…Wameedh

To my Children

moustafa, hussain, zaid

the Harvest of my life.. To everyone

who supported me…

Hawraa 2022

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Hawraa /2022

Summary

The present study was conducted at the College of Sciences in the University of Babylon for the period from February 2021 to October 2021 on laboratory rats for possibility of using garlic extract and loaded on chitosan nanoparticles in alleviating of anemia induced by phenylhydrazine, in adult male rats.

Garlic (*Allium sativum*) extract was purchased from Beijing (China). Alcoholic garlic extract were analyzed by GC-MS, and it was found that there were 20 compounds of garlic extract in different proportions, most of which were Allyl disulfide (66.7%), Diallyl disulfide(10.01%), Disulfide, methyl 2-propenyl (8.87%),3-Vinyl-1,2-dithiacyclohex (3.88%), 6,6-Dimethyl-2-methylenebicyclo[3.1.1]heptane(3%), Methoxy acetic acid (2.44%), and 2,2-Dimthyl-3-methylenbicyclo[2.2.1]heptane (2.34%). After that, the garlic were loaded on chitosan nanoparticles, and their characterization was tested after loading by particle size analysis, which revealed that the size of the garlic extract particles was 11.6 nm, while the size after loading was (25.9) nm.

In Zeta potential analysis, the surface charge of chitosan ($^{+}$30.04mV), TPP ($^{-}$19.62mV), garlic extract ($^{+}$28.80mV), garlic extract loaded on chitosan ($^{+}$48.43mV).Fourier transform infrared spectroscopy showed the presence of many functional groups in the materials (OH, NH, CH,C–O, C=C, C=O, C–N). As for TEM, chitosan nanoparticles appeared with adsorbed layers on the surface representing the materials, where garlic extract appeared as particles with rough surfaces in rounded or spherical to irregular shapes.

The study was conducted on (60) male adult rats were devided to six groups (10 rats per group). Group negative control(NC): animals in this group healthy without any treatment as negative control, Group T1 : animals in this group were injected intraperitonial with two dose 20mg/kg

at 6P.m. and 9A.m. of phynelhydrazine to induced anemia as positive group, Group T2 **:** animals in this group healthy were treated daily with 34.5 mg/kg of garlic extract, Group T3 : animals in this group were injected intraperitonial with two dose 20mg/kg at 6P.m. and 9A.m. of phynelhydrazine to induced anemia and treated daily with 34.5 mg/kg of garlic extract, Group T4 : animals in this group healthy were treated daily with 34.5 mg/kg of garlic loaded on chitosan nanoparticles CNP-G, and Group T5 : animals in this group were injected intraperitonial with two dose 20mg/kg at 6P.m. and 9A.m. of phynelhydrazine to induced anemia and treated daily with 34.5 mg/kg of garlic loaded on chitosan nanoparticles.

In addition to, the animals were sacrificed under deep anesthesia via xylazine - ketamine and blood samples were collected by heart puncture and femur bone were isolated for histological section. Rats expose to phenylhydrazine showed a significant decrease in hematological parameters represented by the total count of red blood cell(RBC), the level of hemoglobin concentration(Hb), and the packed cell volume (PCV) and led to an increase in the mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration(MCHC), and white blood cell (WBC). Also the platelets and erythrocyte sedimentation rate showed significant increase in anemic group when compared with control group. After treatment in (T2,T3,T4, and T5) the platelet and erythrocyte sedimentation rate recorded clear diminished. The serum total protein and albumin showed significantly decreased in anemic group but fibrinogen recorded significantly increase in anemic group as compared with control group. When rats received garlic extract and garlic loaded chitosan nanoparticles reported increase in the blood electrolytes, potassium and iron showed significantly increase in anemic group as compared with control group. While, sodium and calcium recorded significantly decreased. After treatment with extract and garlic

loaded chitosan nanoparticles the K^+ and Fe^+ decrease in all treatment groups and Na^+, Ca^{++} increased in all treatment groups.

Induction of anemia in rats significantly decreased in total antioxidant capacity and increase in malonaldehyde, after treatment rats with garlic extract and garlic loaded chitosan nanoparticles recorded significantly increase in total antioxidant capacity and decrease in malonaldehyde. The serum hemopexin data a significant reduction in positive control group when compared with negative control group, while treatment groups showed a highly significant elevation, erythropoitein showed significant increase in anemic group but recorded decrease in treatment groups, high VB12 in serum of treated group that received garlic loaded chitosan nanoparticles as compared with anemic group ,while T2, T3, and T4 that received garlic extract and garlic loaded chitosan nanoparticles respectively reported non significant different as compared with negative control group. On other aspect our data appear a significant elevation in serum cyclooxygenase in anemia group as compared with control group and among treatment groups. While a significant decrease in T5 and T4 that (received CNP-G) respectively, of serum amyloid A when compared with anemic group, and T2 and T3 (that received garlic extract).

The histological section of bone marrow showed by increase adipose tissue within bone marrow in anemic group, after treatment rats(T2,T3,T4, and T5) with garlic extract and garlic loaded chitosan nanoparticles recorded significantly decrease adipose tissue.

Concerning conclusions, the current study recorded that garlic extract and garlic loaded chitosan nanoparticles was the effective in reducing the anemia. On the other hand, it can be concluded that nanoloading had an important role in increasing the effectiveness of therapeutic material (garlic) in healthy and anemic rats according to the original properties of the material.

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List of Abbreviations

Abbreviation	Meaning
AG	Aged garlic
AI	Anemia of inflammation
Ca^{++}	Calcium
CVD	Cardiovascular disease
CS	Chitosan
CNP	Chitosan nanoparticles
CNP-G	Chitosan nanoparticles- garlic extract
CBC	Complete blood count
C. Area	Compound area
COX-1	Cyclooxygenase
DADS	Diallyl disulfide
DATS	Diallyl trisulfide
EDTA	Disodium ethylene diaminetetraacetate
ELISA	Enzyme linked immunsorbent assay
ESR	Erythrocyte sedimentation rate
EPO	Erythropoietin
FTIR	Fourier transform infrared spectroscopy
FBC	Full blood count
GC-MS	Gas Chromatography- Mass Spectrometry
G6PD	Glucose-6-phosphate dehydrogenase
GSH	Glutathione
g/dl	Gram/ deciliter
gm/L	Gram/ litter
H&E	Haemtoxylin and Eosin

Hb	Hemoglobin
HPX	Hemopexin
HDL	High-density lipoprotein
H_2O_2	Hydrogen peroxide
ID	Iron deficiency
IDA	Iron deficiency anemia
LSD	Least Significant difference
MDA	Malondyaldehyde
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular value
µl	Microlitter
mmol/L	Milli mole/ litter
ng/dl	Nano gram/ deciliter
O.D	Optical density
PCV	Packed cell volume
PHZ	Phynelhydrazine
K^+	Potassium
ROS	Reactive oxygen species
rHuEPO	Recombinant human erythropoietin
RBCs	Red blood corpuscular
Rt	Retention time
RT	Room temperature
SAC	S- allycysteine
SAMC	S-allyl-mercaptocysteine
SAA	Serum amyloid A
Na^+	Sodium
NaOH	Sodium hydroxide
STPP or TPP	Sodium tripolyphosphate
SOD	Superoxide dismutase
T-AOC	Total antioxidant capacity
TIC	Total ion chromatogram
TSP	Total serum protein
TEM	Transmission Electron Microscope
TNF	Tumor necrosis factor
VB12	Vitamin B12
WBC	White blood cell
WHO	World health organization

بسم الله الرحمن الرحيم

﴿ فَتَعَالَى اللَّهُ الْمَلِكُ الْحَقُّ وَلَا تَعْجَلْ بِالْقُرْآنِ مِن قَبْلِ أَن
يُقْضَى إِلَيْكَ وَحْيُهُ وَقُل رَّبِّ زِدْنِي عِلْمًا ﴾

(صدق الله العلي العظيم)

[سورة النور: الايه 114]

1. Introduction

Nanotechnology has played a major role in recent years in the field of biomedicine (Marcano *et al*., 2018), the using of nanoparticles increased the absorption of therapeutic substances and their effects, in addition to improving their efficiency level and their effective dose (Bhavsar *et al*., 2017).

On the other hand, for several subsequent research in recent years, chitosan dependent nanoparticles have become a subject of wide interest due to the biological, chemical, and physical properties they carry with widely used activities that have given them promising roles in the medical field, such as bioactivity, biodegradability, non-toxicity, biocompatibility, low immunogenicity, and high poly-cationic characteristics, which give these particles the safety and efficiency characteristics (Balsam *et al*., 2019).

As a species in the genus *Allium*, garlic (*Allium sativum* L.) is native to Northeastern Iran and Central Asia, which is globally utilized to prepare a variety of dishes with a history of several thousand years (Singh and Singh, 2019). It consists of many bioactive compounds which are primarily sulfur compounds (such as ajoene, allicin, Sallylcysteine (SAC), diallyl sulfide (DAS), diallyl trisulfide (DATS) and diallyl disulfide (DADS)); it is also rich in minerals (such as potassium, iron, zinc, calcium, sulfur, magnesium, manganese and selenium). In addition, it is considered to be one of the abundant sources for phenolic compounds in commonly consumed vegetables (Singh and Singh, 2019).

Anemia a condition in which hemoglobin (Hb) concentration and/or red blood cell (RBC) numbers are lower than normal and insufficient to meet an individual’s physiological needs, anemia is associated with increased morbidity and mortality in women and children,

poor birth outcomes, decreased work productivity in adults, and impaired cognitive and behavioral development in children. (Chaparro and Suchdev, 2019).

Antioxidants represent a form of opposition to oxidants. Antioxidants are natural or synthetic substances that may prevent or delay damage of cell caused by oxidants (ROS, RNS, free radicals, other unstable molecules) (Azeez *et al*., 2017), while malondyaldehyde its degradation of lipid hydroperoxides results in the production of bioactive aldehydes. (Ates *et al*., 2018).

Hemopexin (HPX) is a glycoprotein formed from a single polypeptide chain, which binds free haem for detoxification and transport. There are no physiological genetic or structural variants known (Griffiths *et al*., 2019). Erythropoietin (EPO) is a glycoprotein hormone mainly expressed by peritubular fibroblasts in the renal cortex. EPO secretion is regulated by hemoglobin (Hgb) levels and tissue oxygenation (pO2) in the outer medulla of the kidney (Kolomansky *et al.*, 2020).

Vitamin B12 (cobalamin) is a general name of several cobalt-containing corrinoid compounds, which are essential for the proper metabolism and function of all animal organs and systems (Allen *et al*., 2018). Cyclooxygenases are an important and thoroughly studied group of enzymes present in two isoforms in mammals: constitutive form cyclooxygenase-1 (COX-1) and an inducible form cyclooxygenase-2 (COX-2) (Leao *et al*.,2020). Serum amyloid A (SAA) is an acute phase protein and inflammation marker. Levels of circulating SAA increase ~1000-fold in response to microbial infection or trauma, and persistently high levels are evident in chronic pathologies such as diabetes mellitus (McEneny *et al*., 2015),

Aim of study

To investigate the therapeutic activity of garlic nanoparticles in improvement of experimentally induced anemia in rats by measuring some physiological and biomarkers parameters as following:-

1. Measurement of the complete blood count(CBC).
2. Evaluating the biochemical parameters(total serum protein, albumin, fibrinogen, and blood electrolytes).
3. Evaluating antioxidant indicators (total antioxidant capacity and malondialdhyde).
4. Determine the cyclooxygenase, hemopexin, erythropoietin, and vitamin B12.
5. Measurement of serum amyloid to determine the toxicity of material nanoparticles.
6. Studying of the histological changes in the femur bone marrow.

2. Literature Review

2.1 Nanotechnology

Science includes the use of nanoparticles for biomedical purposes, which is generally called Nanotechnology (Ahluwalia *et al*., 2018). The application of nanoparticles and their encapsulation processes have attracted attention in the field of pharmacological research and biomedicine, as working on nanoparticles of small size has improved the ability of treatments to cure patients by improving the vital distribution, delivering the drug to a special location orally, subcutaneously, or intravenously (Wong *et al.*, 2017; Patil *et al*., 2019).

There is a list of natural or chemical nanomaterials that can be used, but it is harmful at certain concentrations, such as minerals which generate toxicity to the body or the environment during production, application, or disposal. Some of them are associated with side effects on the organism, as in the use of silver, iron oxide, and cerium oxide, which show a high mortality rate, while natural materials such as chitosan showed an efficient and safe workability as a nanomaterial due to its good properties such as antimicrobial and antioxidant, in addition to being carbohydrates that has poor toxicity, abundance sources, biocompatibility and biodegradability (Nayak *et al*.,2016; Kuen *et al*.,2017), also environmental friendliness, low cost and increased efficiency in many applications such as transmission, genetic delivery and antimicrobial agents, targeting of cancer cells and therapeutic methods (Vallet-Regi *et al*., 2018; Huang *et al*., 2018; Wu *et al*., 2018). Biopolymers applications such as cellulose, collagen, starch, gelatin, albumin, and chitosan-dependent nanomaterials have added favorable properties to nanoparticles (Rasool *et al.,* 2016 ; Yang *et al*., 2018).

2.2 Chitosan

Chitosan (CS) is a naturally multi-nitrogen polysaccharide comprising a cationic hydrophilic polymer with high alkalinity, biocompatible, non-toxic, white solid granules used as a carrier to prepare nanoparticles for loaded with substances (Carter and Puig-Sellart, 2016). It consists of Niacetyl-D-glucosamine (acetylated) and beta(1-4)-linked D-glucosamine (deacetylated) units, where the most important characteristic of its positive nature is that it can form ionic complexes that have no solubility in water (Hardy *et al*., 2018).

Chitosan is produced from the alkaline hydrolysis of chitin (Ch) by removing the acetate moiety figure(2-1), where removing the proteins and minerals from the chitin obtained from the cell walls of fungi, rigid structures of invertebrate animals, the shells of crustaceans, orthopoda, and insects, then partly N-deacetylated to purify the chitosan as natural amino polysaccharides (Motiei *et al.*, 2017; Jabar, 2020).

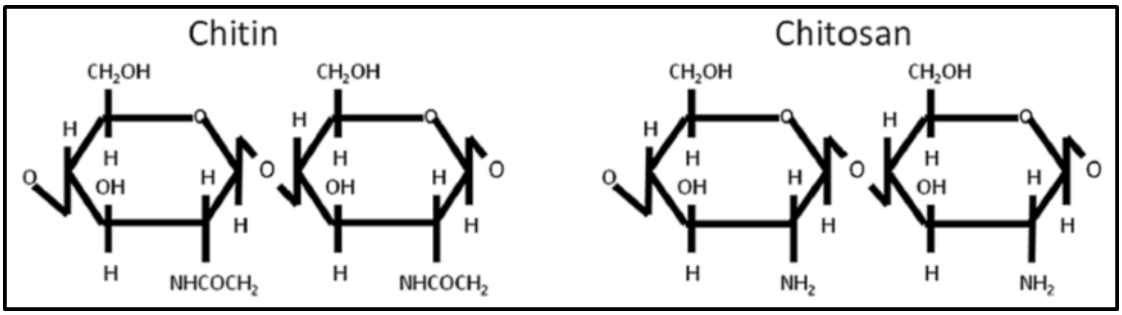

Figure 2-1: Structure of chitin and chitosan (Motiei *et al.*, 2017).

Chitosan is abundant with functional groups of hydroxyl (OH) and amine (NH2) that have been used to create cross-chemical bonds (Balsam *et al*.,2019), while Divya and Jisha, (2018) mentioned that chitosan consists of three effective groups, which are the amine groups, primary hydroxyl group, and secondary hydroxyl group at the carbon sites C2, C3, and C6, and this design allows chitosan to have desirable traits, including modification and binding to other substances (Bhavsar *et al*., 2017), but it

is affected by other such as strong acids that cause depolymerization of chitosan (Divya and Jisha,2018).

It's important to note, chitosan can biodegrade into simple, non-toxic sugars such as glucosamine and N-acetylglucosamine by special enzymes such as bacterial chitinase or lysozyme chitosases, this provides an appropriate case for medical applications that have shown great success in the field of chitosan-based nanoparticles and their derivatives in antioxidant activity, antimicrobial activity, and improving overall stability in biological materials, including fluids (Ska *et al*., 2017; Mohebbi *et al*., 2019).

In the pharmacological field and drug delivery, the nanoparticles act as plates used to load the drug. According to this, the chitosan is considered as one of the most important natural polymers that is used in multiple fields because of its positive effects and its solubility in the aqueous medium, which leads to more accurate regulation and distribution of the drug in a mechanism that depends on its molecular weight, then chitosan is excreted immediately through the kidneys after delivery of the drug (Bellich *et al*., 2016).

The loading of therapeutic substance on chitosan have added protection from the effect of decomposition enzymes inside the body with the absorption and bioavailability in the cell (Senapati *et al*.,2018), where there is a beneficial property of chitosan to prolong the retention period of drugs inside the body by encapsulating protein and peptide drugs without changing their effectiveness due to the loss of an organic solvent and to prevent their digestion by proteolytic enzymes intravenously, as the size of the particles, surface charge and hydrophobic feature are factors that have an effect on the effectiveness of chitosan and its derivatives (He *et al*., 2010).

2.3 Garlic

Garlic (*Allium sativum* L.) is a bulb belonging to the Liliaceae family, which belongs to the Allium genus (Ramírez-Concepción *et al*., 2016), it is important from the economic point of view and especially for its nutraceutical properties and its benefits for human health. *Allium sativum* L. is a species native to Central Asia (west of the Himalayas) that was cultivated in China, Mesopotamia and Egypt 5000 years ago, it was brought by the Spaniards, at the end of the XV th century, garlic would enter the American continent as a seasoning product (Wu *et al*., 2015). Many members of the genus Allium, including about 700 species, have been recognized as rich sources of biologically active secondary metabolites in addition to their antioxidant properties (Herrera *et al*., 2014; Upadhyay, 2016).

Garlic is one of the oldest vegetative propagated horticultural crops. The edible part of garlic is its fresh bulbs. The bulb, its main body part, is also called the 'garlic head', while each of the bulb's segments is referred to as a 'garlic clove'. Garlic has been used since ancient times not only to flavor foods, but as a medicinal plant (Morales-González *et al*., 2019). Most of its health benefits are due to the presence of allicin molecules(Touloupakis and Ghanotakis,2010; Varga-Visi *et al*., 2019).

The culinary, medicinal, and insecticidal properties of garlic are related to the large variety of molecules it contains, including protein, fat, carbohydrates, fiber, ash, sulfur compounds, essential oils, and minerals such as potassium (K), phosphorus (P), magnesium (Mg), sodium (Na), calcium (Ca), and iron (Fe). Garlic is therefore beneficial to human health, its strong and astringent taste is due to its organosulfur compounds, which have been associated with its nutraceutical properties (Frankel *et al*., 2016).

The eating raw, garlic can be beneficial in preventing some diseases such as strains of the common cold, cardio-vascular diseases, and high blood pressure (Shafiur, 2007). Therefore, the spread of information related to the preventive and curative properties of garlic along with its benefits to help fight various diseases and its benefits to human health, have greatly increased the consumption of this species (Fratianni *et al*., 2016).

2.3.1 Protective and physiological properties of garlic

The major physiological role of garlic its antimicrobial, anticancer, antioxidant, immune boosting, antidiabetic, hepatoprotective, antifibri-nolytic and antiplatelet aggregatory activity and its potential role in preventing cardiovascular diseases (Santhosha *et al*., 2013). Various treatments have been found for different types of garlic products. Fresh garlic is the product with the best antioxidant and antimicrobial effectiveness, while natural food additives are better than synthetic ones. Garlic oil contains antioxidant compounds as well as pro-oxidants that produce a number of natural protective substances, these substances intervene in various metabolic processes, practically blocking the harmful effect of free radicals generated in specific situations, such as physical stress, and malignant tumors (Kim *et al*., 2010).

Antioxidants are usually found in food or in the human body in small concentrations compared to oxidizable substrates, the flavonoid content in garlic, for example, is between 8.8 mg/100 g and 2.3 mg/100 g in a fresh sample (Somman and Siwarungson, 2015).

The physiological effects of garlic include lowering basal metabolism and reducing metabolism disorders caused by fast absorption carbohydrates, these factors help to strengthen human health by significantly decreasing morbidity and mortality. The inclusion of new approaches and health-protective processes represents a new opportunity

for public health, which can broaden and enrich this field of knowledge. (Espinoza *et al*., 2020).

2.3.2 Antioxidant activity of garlic

Asdaq and Inamdar (2011) reported that the frequent garlic intake promotes internal antioxidant activities and reduces oxidative adverse effects either by increasing the endogenous antioxidant synthesis or reducing the production of oxidizers such as oxygen-free radical species (ORS).

As ROS seems to be at the core of many ailments, it is justified to assume that the antioxidant effect of garlic might be through modulation of ROS, increasing glutathione and cellular antioxidant enzymes (Shokrzadeh and Ebadi 2006). Moreover, garlic extract was found to increase the activities of some antioxidant enzymes (e.g., superoxide dismutase (SOD)) and decrease glutathione peroxidase (GSH-Px) in hepatic tissues of rats (Jang *et al*., 2017).

Additionally, garlic acted by stimulating the expression of different antioxidant enzymes, namely glutamate-cysteine ligase modifier (GCLM) and heme oxygenase-1 (HO-1) subunit by the nuclear factor erythrobia-2 related factor 2 (Nrf2)-antioxidant response element (ARE) pathway that is responsible for human endothelial cells protection against oxidative stress (Liu *et al*., 2018). Alliin, the major compound isolated from AGE, showing wide-spectrum antioxidant activities by controlling ROS generation and preventing mitogen-activated protein kinase (MAPK). Moreover, it was reported to prevent ROS production by inhibiting NADPH oxidase 1, and thus, inhibiting the osteoclast fusion caused by receptor activator of nuclear factor-kappa B ligand (RANKL) (Chen *et al*., 2016).

Allicin, DADS, and DATS are the main antioxidative compounds that showed an antioxidant effect in lower doses at the physiological level

(Gruhlke *et al*., 2010). Saponins extracted from garlic were reported to scavenge intracellular ROS and protect mouse-derived C2C12 myoblasts towards growth inhibition and H2O2-induced DNA damage (Shang *et al*., 2019).

2.3.3 Medicinal important of garlic

Complementary medicine is gaining importance, mainly in ethnobotany, phytotherapy, and phytochemistry, garlic has been clinically used in many traditional medical systems since ancient times, mainly for treating and preventing diseases (Baliga *et al*., 2013). Garlic has also been shown to strengthen the immune and anti-tumor systems, and the antioxidant activity of garlic polysaccharide protects the body against the harmful effects of free radicals or hydroxyl (Chen and Huang, 2020; Cheng *et al*., 2020; Upadhyay, 2016).

A healthy diet with plenty of functional foods made with garlic has been found as beneficial to human health. Fresh garlic can alter anticoagulant levels in the blood and stimulate activity in all the digestive and respiratory organs (Lee *et al*., 2019). Garlic helps the liver (Abdel-Daim *et al*., 2015) and gall bladder function allium properly; it defends them against gut infections (Gatt *et al*., 2015) and problems caused by the decomposition of intestinal bacteria (Leyva *et al*., 2016).

Garlic lowers blood pressure (Cicero and Borghi, 2013) and stimulates the circulatory capacity of the heart. Furthermore, it has antihypertensive, hypolipidemic, antiathero-genic, anticarcinogenic, antitumor, antiaggregant, fibrinolytic, antianemic, antimicrobial, antifungal, and immunomodulatory properties. According to Moneim (2015), treatment using macerated garlic extract can help prevent neurodegeneration by alleviating stress.

Iranloye (2002) shown that daily feeding of rats with 200mg/kg garlic juice slightly increases the haemoglobin concentration, the red cell

count and the packed cell volume (PCV). In addition, the increased in these parameters may be explained as garlic plant is considered an active oxygen scavenger. It is thus possible that garlic components compete with hemoglobin in the red blood cell for oxygen resulting in hypoxia which then stimulates hemoglobin synthesis and red blood cell production (Suha, 2014).

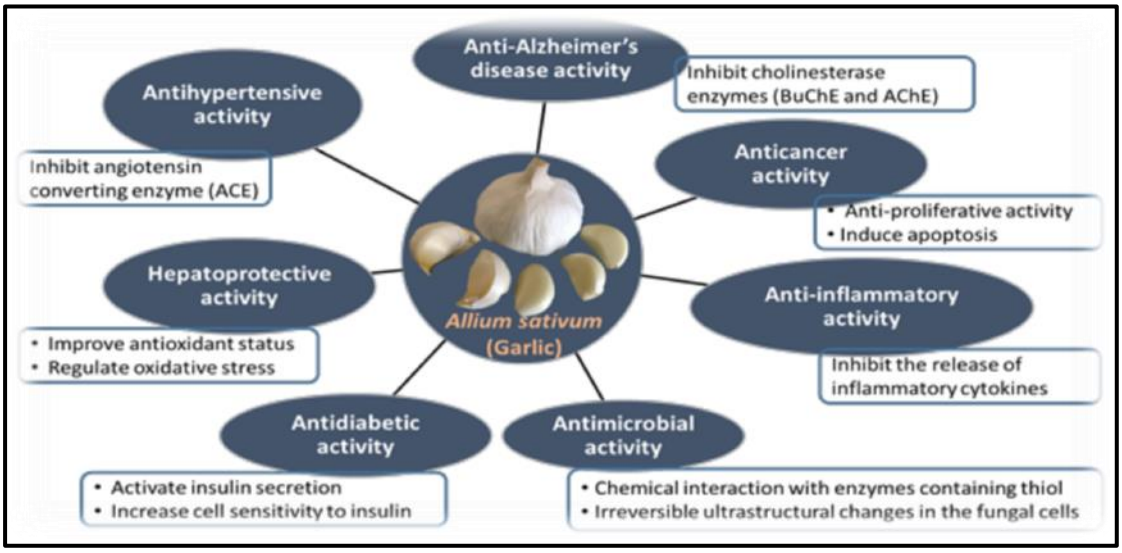

Figure 2-2: Schematic representation of different pharmacological activities of garlic (Allium sativum) and their mechanisms.(Batiha *et al.*, 2020).

2.4 Anemia

Anemia is alternately defined as a reduced number of circulating red blood cells(RBCs) (Schreir, 2018) or a condition in which the number of RBCs (and subsequently their oxygen-carrying apacity) is insufficient to meet physiologic needs (WHO, 2011), anemia can also be diagnosed using RBC count, mean corpuscular volume, blood reticulocyte count, blood film analysis, or Hb electrophoresis (Balarajan *et al*., 2011).

At a biological level, anemia develops because of an imbalance in erythrocyte loss relative to production; this can be due to ineffective or deficient erythropoiesis (e.g., from nutritional deficiencies, inflammation, or genetic Hb disorders) and/or excessive loss of erythrocytes (due to hemolysis, blood loss, or both). Anemia is frequently classified based on

the biological mechanism of causation (e.g., iron deficiency anemia, hemolytic anemia, and inflammation anemia) and/or the RBC morphology (Braunstein, 2017).

Furthermore, as anemia may have multiple causes, even in the same individual, hematological manifestations of a particular cause can be masked by another. For example, the hallmark of anemia caused by vitamin B12 or folate deficiencies is macrocytic anemia. Concomitant ID, which causes microcytosis, may mask entirely the effects of the B12 or folate deficiency (Hoffman *et al*., 2015).

Figure (2-3) is a conceptual model of the etiology of anemia identifying how distal factors contribute to more proximate determinants of anemia, such as food insecurity, clean water, and sanitation, and, ultimately, the most immediate causes of anemia (e.g., nutritional deficiencies, disease, inflammation, and Hb disorders) (Pasricha *et al*., 2013 ; Namaster *et al*., 2017).

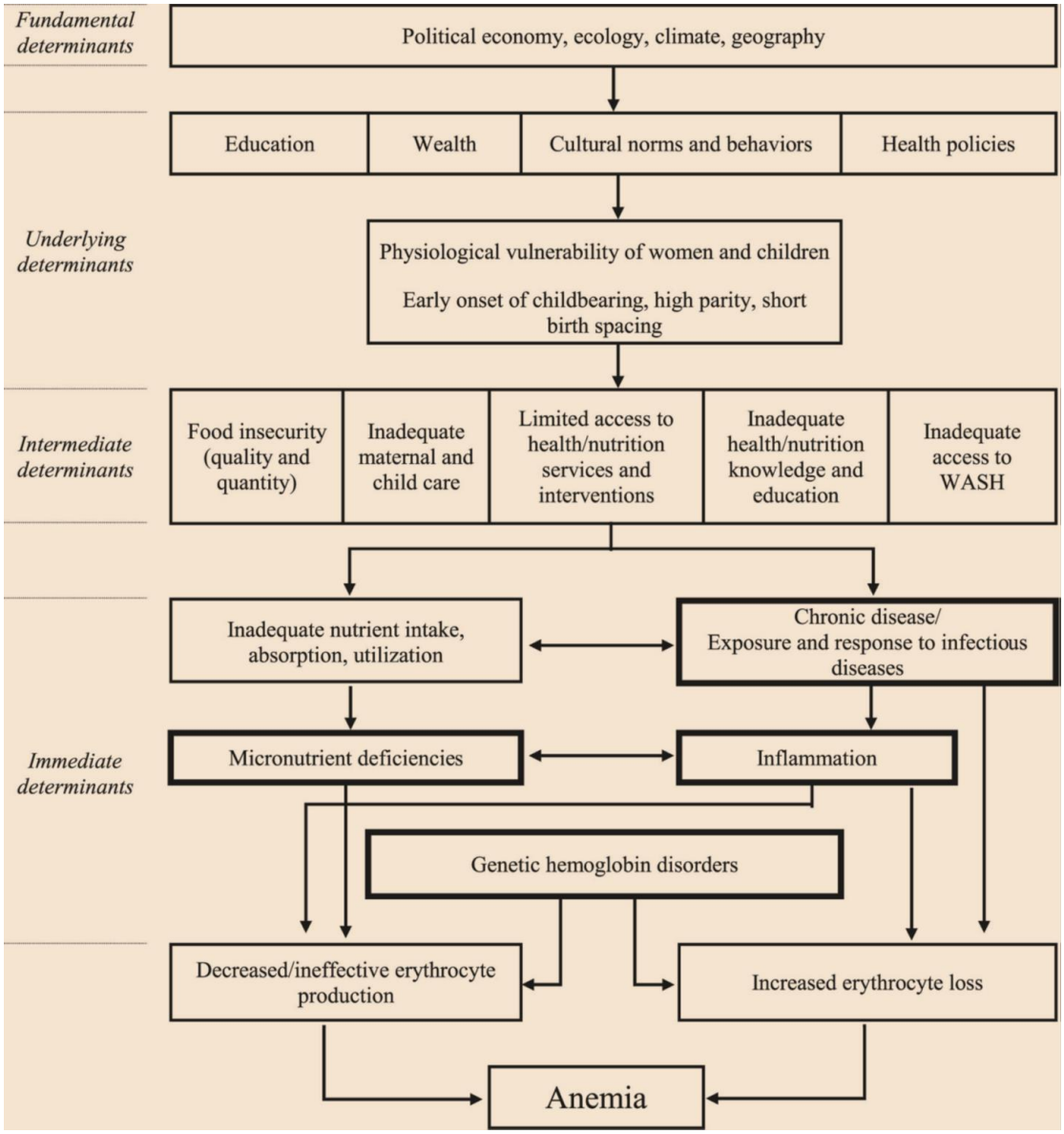

Figure 2-3: A conceptual model of anemia etiology (Pasricha *et al*., 2013).

2.5 Phenyl hydrazine (PHZ)

Phenylhydrazine (Hydrazinobenzene) the Chemical formula C6H8N2,it is mainly used as a chemical intermediate in the pharmaceutical, agrochemical, and chemical industries, Phenyl hydrazine was mainly used for experimental induction of anemia in animals, PHZ decreases Hemoglobin concentration, RBC (Red Blood Cell) count and PCV (Packed Cell Volume) whereas increases the MCV (Mean Cell Volume), MCH (Mean Cell Hemoglobin), MCHC (Mean Corpuscular Hemoglobin Concentration) and extramedular haematopoiesis in the spleen and liver (Singh *et al*., 2014).

Phenylhydrazine is absorbed by the inhalation, oral and dermal routs hemotoxicant PHZ causes oxidative stress within erythrocytes resulting oxidation of oxyhemoglobin leading to the formation of methemoglobin which is subsequently converted into irreversible hemichromes that lead to the precipitation of hemoglobin in the form of Heinz bodies. PHZ causes damage in skeletal protein, lipid peroxidation, ATP depletion, cation imbalances, and reduced membrane deformability (Kolawole *et al.*, 2017).

2.6 Antioxidant and oxidation status

2.6.1 Total antioxidant capacity (T-AOC)

Antioxidants represent a form of opposition to oxidants. Antioxidants are natural or synthetic substances that may prevent or delay damage of cell caused by oxidants (ROS, RNS, free radicals, other unstable molecules) (Azeez *et al*., 2017), defined antioxidant as any substance that delays, prevents, or removes oxidative damage to a target molecule (Jing *et al*., 2011). In order for the substance to be considered as an antioxidant, it must be active at low concentration (phenolic antioxidants often loose activity at high concentration and act as

prooxidant), its amount needs to be satisfactory high to deactivate the target molecule, it must react with oxygen or nitrogen free radicals, and the final product of the reaction should be less toxic than removed radical. There is no universal antioxidant, as different antioxidants react with different reactive species by various mechanisms, at various locations and protect specific molecular targets (Bedlovicova *et al*., 2020). Generally, the antioxidant defense can become active either by in vivo processes (synthesis of intracellular enzymes-superoxide dismutases, superoxide reductases, peroxiredoxins, glutathione peroxidases, catalases, peptides-glutathione; or in the form of extracellular antioxidant defenses-synthesis of transferrin, erythrocytes, albumin, urate, glucose; low-molecular mass agents-bilirubin, -keto acids, melatonin, lipoic acid, coenzyme Q, uric acid) or by supplying missing substances in the form of a diet (vitamins-C, E, A, D, riboflavin, thiamine, niacin, pyridoxine, carotenoids, flavonoids, polyphenols, amino acids, folic acid, phytoalexins, elements Se, Fe, Zn, Mg) (Jing *et al*., 2011; Haliwell and Gutteridge, 2015).

The antioxidant property exhibited by whole garlic and aged garlic extract also enhances the serum levels of two antioxidant enzymes, catalase and glutathione peroxidase. S-allyl cysteine, a component of garlic also confirmed significant antioxidant effects. The sulfur compounds found in fresh garlic appear to be nearly 1000 times more potent as antioxidants than crude, aged garlic extract. Garlic (both the homogenate of 10% in physiological saline solution and its supernatant) was able to reduce the radicals present in cigarette smoke .Furthermore, allicin being another component that is abundant in dried garlic and is formed when garlic is crushed. According to recent studies, allicin decomposes to form sulfenic acid, a potent antioxidant (Chidinma *et al*., 2019).

2.6.2 Malondialdehyde (MDA)

The degradation of lipid hydroperoxides results in the production of bioactive aldehydes. One of the most important of these bioactive aldehydes is MDA. Because of its easy reaction with thiobarbituric acid (TBA) ,MDA has been in use for many years as a suitable biomarker for lipid peroxidation of omega-3 and omega-6 fatty acids (Ates *et al*., 2018).

Because MDA is one of the most widespread and reliable markers of oxidative stress in clinical situations and because of its high reactivity and toxicity, this molecule is highly popular in the biomedical research community. MDA is used to predict damage relative to reactive oxygen species (Giera *et al*.,2012). MDA is one of the low molecular weight end products of lipid hydroperoxide degradation. It is one of the final products of lipid peroxidation and is often used as a biomarker of the oxidative stress. MDA is an important reactive effect due to peroxidation of biological membranes. The most common method used to assess MDA production is thiobarbituric acid reactive substances (TBARS) marker (Ayala *et al*., 2014).

2.7 Biomarkers to evaluation blood (HPX, EPO, VB12, and COX-1)

2.7.1 Hemopexin (HPX)

Hemopexin (HPX) is a glycoprotein formed from a single polypeptide chain as in figure (2-4), which binds free haem for detoxification and transport. There are no physiological genetic or structural variants known (Griffiths *et al*., 2019). Clearance is primarily by hepatocytes,but the appropriate receptor (LRP1) is present on many cell types (Tolosano *et al*., 2010; Allmendinger *et al*., 2012).

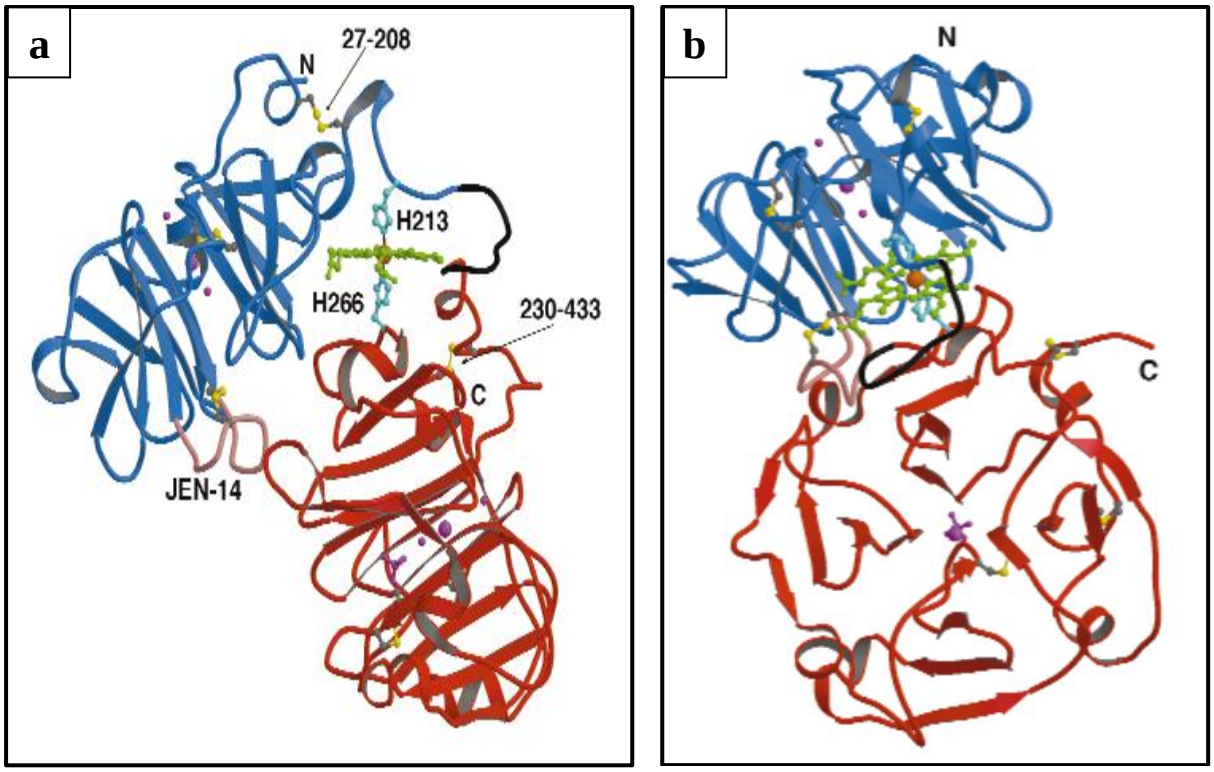

Figure (2-4) Structure of the heme-complex. a, b, The b-propeller domains are colored blue (N-domain) and red (C-domain),the heme group green, coordinating histidine residues cyan and disulfide bonds yellow (disulfides that close the two b-propellers are labeled). The loop JEN-14 is pink, and the black portion of the interdomain linker is a flexible section lacking interpretable electron density (219–221 in native Hpx,216–221 in deglycosylated Hpx); The ions in the central tunnel of each propeller (two Na+ and one Cl- in each domain, and a phosphate in the C-domain only) are purple.(Paoli *et al.*, 1999).

HPX is synthesised primarily in the liver; however, there is expression by neurons, astrocytes, ventricular ependymal cells, kidney, skeletal muscle and retina. HPX is also regulated by IL-6 (Griffiths *et al*., 2019). In hemolytic conditions, occurring e.g., in malaria, sickle cell disease, and thalassemia, erythrocytes undergo lysis and large amounts of hemoglobin (≥20 μM) enter the blood stream (Dutra *et al*., 2014; Martins *et al*., 2016).

In healthy patients, released hemoglobin is directly bound by haptoglobin and transported to hepatocytes and macrophages for its degradation. This protective mechanism, however, can collapse when the scavenging capacity of haptoglobin is exhausted, hemoglobin is oxidized

to methemoglobin, and heme (Fe(III) protoporphyrin IX) is released into the blood (Detzel *et al*., 2020).

As a consequence of ROS production, oxidative damage may occur to proteins, carbohydrates, lipids, and nucleic acids4. In addition, heme itself can act as regulator by transiently binding to proteins (WiBbrock *et al*., 2019). It can directly alter the function of several plasma proteins that are part of the immune system e.g., IgG, C1q, and TLR4, and exacerbate other pathologies (Roumenina *et al*., 2011). Consequences of the accumulation of labile heme are hepatic injuries in sepsis, nephropathy in hemolysis patients, and leukocyte and reticulocyte adhesion to endothelial cells (Englert *et al*., 2019; Wang *et al*., 2019).

2.7.2 Erythropoietin (EPO)

Erythropoietin (EPO) is a glycoprotein hormone as in figure (2-5), mainly expressed by peritubular fibroblasts in the renal cortex, EPO secretion is regulated by hemoglobin (Hgb) levels and tissue oxygenation (pO2) in the outer medulla of the kidney. The synthesis of EPO is regulated at the transcriptional level, i.e., a low tissue pO2 leads to up-regulation of EPO synthesis and consequent stimulation of erythrocyte production. (Kolomansky *et al.*, 2020).

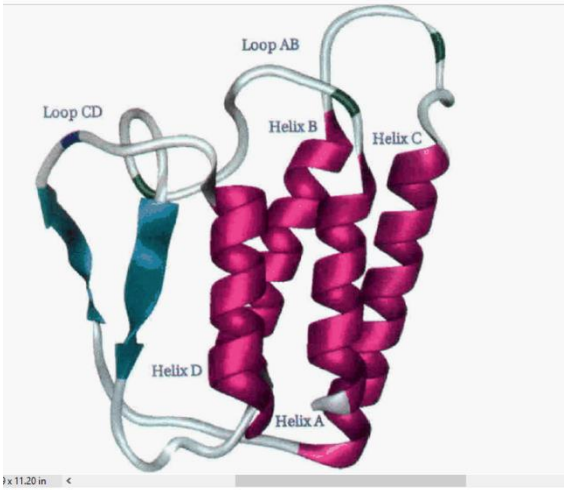

Figure 2-5 Model of the three-dimensional structure of erythropoietin(Boissel *et al*., 1993).

The normal concentration of EPO in rat plasma is about 25 U/l. After acute hypoxic stress, the plasma EPO concentration rises within 1.5-2 hours and can increase up to 100-fold within 18 h, recombinant human EPO (rHuEPO) is used in clinical practice for the treatment of several types of anemia (Cariou *et al*., 2016). It has been long recognized by clinicians that, while low Hgb levels lead to anemia, excessive Hgb levels can increase mortality as a result of cardiovascular and thromboembolic events. (Giagounidis, 2017).

In addition, detecting the expression of EPO-R at the protein level is challenging due to the lack of a specific antibody. EPO both increases bone resorption and decreases bone formation, leading to extensive bone loss within two weeks of treatment (Rauner *et al*., 2016). Importantly, endogenously high EPO levels, either due to induced anemia or constitutive overexpression of this hormone (Hiram-Bab *et al*., 2017) (both in murine models), also lead to significant bone loss. In human subjects (elderly men with normal renal function) high plasma levels of EPO were associated with increased fracture risk (Kristjansdottir *et al*., 2020).

2.7.3 Vitamin B12 (VB12)

Vitamin B12 (cobalamin) is a general name of several cobalt-containing corrinoid compounds,which are essential for the proper metabolism and function of all animal organs and systems. In the human body, only two forms are biologically active, adenosylcobalamin and methylocobalamin, functioning as coenzymes in radical-induced rearrangements and methylation processes, respectively (Allen *et al*., 2018).

Adenosylcobalamin is a cofactor for mitochondrial methylmalonyl-CoA mutase and it is essential for the metabolism of fatty acids and ketogenic amino acids. Methylcobalamin is a cofactor for cytosolic

methionine synthase that catalyzes the conversion of homocysteine to methionine. Methionine is then converted to S-adenosylmethionine, a universal methyl group donor for methylation reactions throughout the body, including the methylation of DNA, RNA and proteins (Herrmann and Obeid, 2012 ; Allen *et al*., 2018).

Cobalamin is a unique vitamin produced exclusively by bacteria. It enters the animal food chain via herbivores, which accumulate cobalamin during intestinal fermentation of grass (performed by certain cobalamin-producing bacteria) (Krzywanski *et al*., 2020). The major physiological action of vitamin B12, relevant to sportsmen, include its involvement in red blood cells formation in bone marrow (O'Leary and Samman, 2010).

Another potentially beneficial actions of vitamin B12 are maintenance of proper immune function, improved transmission of neural signals and synthesis of neurotransmitters and creatine (Gleeson and Williams, 2013; Pyne *et al.*, 2014). Athletes and coaches believe that enhanced red blood cell parameters are desirable for optimal performance, therefore hemoglobin concentration is their favorite biomarker. Thus, B12 is a commonly used supplement in many branches of sport and in addition, many athletes and coaches strongly insist on the unjustified administration of vitamin B12, especially as injections (Eskici and Ersoy, 2016).

2.7.4 Cyclooxygenase (COX-1)

Cyclooxygenases are an important and thoroughly studied group of enzymes present in two isoforms in mammals: constitutive form cyclooxygenase-1 (COX-1) and an inducible form cyclooxygenase-2 (COX-2) (Leao *et al*.,2020).

The COX-1 enzyme is expressed in most tissues and is responsible for maintaining homeostasis and production of prostaglandins (PGs) . COX-2 is found predominantly in the brain, renal, and endothelial cells

and is significantly increased through various acute and chronic inflammatory infections (Cámara-Lemarroy *et al*.,2010).

The two isoforms COX-1 and COX-2 catalyzing the synthesis of inflammatory molecules are almost similar in weight with 60–65% sequence similarity; however, they differ in their localization and expression. While COX-1 is constitutively expressed in nearly all tissues, COX-2 is an inducible enzyme primarily localized to immune cells such as macrophages and leucocytes and upregulated in pathological conditions (Rawat *et al*., 2019).

2.8 Biomarker to evaluation toxicity

2.8.1 Serum amyloid A (SAA)

Serum amyloid A (SAA) proteins are small (104 amino acids in human) and remarkably well-conserved in mammalian evolution (George and Sack, 2020). SAA an acute phase protein and inflammation marker. Levels of circulating SAA increase ~1000-fold in response to microbial infection or trauma, and persistently high levels are evident in chronic pathologies such as diabetes mellitus (McEneny *et al*., 2015),rheumatic disorders (Hwang *et al*., 2016), various cancers (Ren *et al*.,2014) and chronic inflammatory disorders such as atherosclerosis (Thompson *et al*.,2015).

The hepatocyte is the primary source of SAA and these cells yield the acute phase protein in response to inflammatory cytokines, including tumor necrosis factor (TNF), and interleukin 1 and 6 (IL-1 and IL-6). In addition, stimulated monocytes/macrophages, vascular smooth muscle cells, and endothelial cells synthesize SAA. Deposits of the acute phase protein are detected in human plaque (Cai *et al*., 2020). Whether SAA in the vascular wall elicits inflammatory responses is presently unclear.

However, the potential for SAA to instigate endothelial (Chami *et al*., 2015) and renal dysfunction has been established (Dieter *et al*., 2016).

Blood levels of SAA are related to CVD risk, however, high-density lipoprotein (HDL) that binds SAA shows an inverse relationship with CVD. Thus, the interaction between HDL and SAA(and indeed the HDL:SAA ratio) may be important in defining the biological actions of SAA (Chami *et al*., 2015).

2.9 Histological evaluation of femur bone marrow

Bone is a dynamic tissue that is continuously resorbed by osteoclasts and neoformed by osteoblasts. The bone remodeling, which is a highly complex process, is under the control of local and systemic factors that all together contribute to bone homeostasis. Besides osteoclasts and osteoblasts, it has been demonstrated that osteocytes, which comprise 90-95% of the total bone cells, play a key role during the bone remodeling cycle (Florencio-Silva *et al.*, 2015).

Osteocytes act as orchestrators producing factors, such as RANKL and sclerostin, that influence osteoclast and osteoblast activities (Gaudio *et al.*, 2020). In the last decade, numerous studies have supported the role of some factors released by osteocytes in the pathogenesis of metabolic bone diseases, but also of rheumatological (Intemann *et al*., 2020) and systemic diseases (Gaudio *et al.*, 2017). Osteoporosis is a common skeletal disorder characterized by compromised bone strength that predisposes patients to an increased risk of fracture (Lorentzon and Cummings, 2015).

Among the secondary forms of osteoporosis, hematological diseases play a very important role. It seems logical to think that, given the close relationships between bone and bone marrow, alterations in the latter can also have a significant impact on bone health. Studies conducted in animal models showed that bone cells interact with

hematopoietic cells, providing a supportive microenvironment needed to maintain erythropoiesis and myelopoiesis (Valderrrabano and Wu, 2019).

Nevertheless, the effects of hematological diseases on bone are not only caused by the close interconnections between bone marrow cells and bone but are also due to a whole series of circulating factors, such as cytokines, that can alter bone turnover, increasing the activity of osteoclasts and/or reducing the action of osteoblasts. There is mounting evidence that anemia per se, that characterizes several hematological diseases, may also be associated with bone fragility (Valderrrabano and Wu, 2019).

Among the hypothesized mechanisms of this association, hypoxia seems to play an important role. In fact, hypoxia is a potent stimulator of erythropoietin production that stimulates osteoclast precursors and induces bone loss (Hiram-Bab *et al.,* 2015). Iron deficiency, which is observed in chronic blood loss, may also affect bone health. Iron, in fact, is an essential cofactor for hydroxylation of prolyl and lysil residues of procollagen and participates in vitamin D metabolism through the cytochromesP450 (Toxqui and Vaquero, 2015). Finally, bone tissue can be affected by systemic complications related to hematological diseases (Mirza and Canalis, 2015).

3. Materials and Methods

3.1 Materials

3.1.1Instruments and tools

Instruments, chemical and kits used in the present study with their suppliers were listed in table (3-1) and table (3-2).

Table 3-1: Instruments and tools used in this study.

NO.	Instruments	Company	Country
1.	Centrifuge	HETTICH	Germany
2.	EDTA tube, Pt tube	AFCO-AFCO	Jordan
3.	ELISA	Biotech	USA
4.	FTIR analysis	TENSOR 27	German
5.	GC-Mass apparatus	SHIMADZU	Japan
6.	Geno tech	AFLO	USA
7.	Horib A	Biomerieux	France
8.	Hotplate stirrer	LP-LABINCO	USA
9.	Light microscope	CYAN	Belgium
10.	Micropipette	Win lab	Germany
11.	Particle size analyzer	BROOK HAVEN	USA
12.	PH meter	HANNA	Romania
13.	Pipette, Beaker, Flask	MARIENFELD	Germany
14.	Sensitive balance	DENVEN	Germany
15.	SHIMADZU 2010	SHIMADZU	Japan
16.	Sonicators	MISONIX	England
17.	Spectrophotometer	VIS-7220G	UK
18.	Transmission Electron Microscope	JEOL LTD	Japan
19.	Vortex, Cool box	GEMMY	Taiwan
20.	Zeta PLUS analyzer	BROOK HAVEN	USA

3.1.2 Chemicals

All the chemicals and the standard kits used in this study were shown in table (3-2).

Table 3-2: Chemicals and kits used through this study.

No.	Chemicals and kits	Company	Country
1.	Calcium, Potassium, Sodium, Iron	Giessediagnostic	Italia
2.	Chitosan	Beijing	China
3.	Formalin	SIGMA-ALO	Germany
4.	Glacial acetic acid	BDH	England
5.	Ketamine	Kepro	Holland
6.	Phenylhydrazine	Thomas Baker	India
7.	Sodium tripolyphosphate	DAEJUNG	Korea
8.	Total protein, Albumin	Giesse diagnostic	Italia
9.	Xylazine	VMD	Belgium
10.	Rat total antioxidant capacity(TAOC)	Bioassay	China
11.	Rat Malondialdehyde (MDA)	Bioassay	China
12.	Rat Hemopexin (HPX)	Bioassay	China
13.	Rat Erythropoietin(EPO)	Bioassay	China
14.	Rat vitamin B12(VB12)	Bioassay	China
15.	Rat cyclooxygenase (COX-1)	Bioassay	China
16.	Rat Serum amyloid A(SAA)	Elabscience	USA

3.2 Methods

3.2.1 GC-MS analysis for garlic extract

To prepare the samples for measuring by Gas Chromatography-Mass Spectroscopy according to (Trezzi *et al*., 2016), 10 mg of dry garlic extract was added to a glass tube containing 5 ml of ethanol and methanol solvent, mixed in a water bath for 30 minutes at 60 ºC, after which 1 µl was injected into GC-MS device in environment and water research laboratory at ministry of science and technology according to the conditions of experiment in table (3-3).

Table 3-3: The condition information of GC-MS used in the experiment

Setting	Condition Values	
GC	Injection temperature	280 ºC
	Column temperature	60 ºC
	Injection mode	Split
	Carrier gas	He (Constant Liner Velocity)
	Liner velocity	46.3 cm/ sec
	Split ratio	10
	Injection volume	1 µ
MS	Ion source temperature	200 ºC
	Interface temperature	280 ºC
	Scan range	35-600 m/z
	Event time	0.50 sec
	Scan speed	1250 µ/ sec

3.2.2 Preparation of nanoparticles

Preparation of nanoparticles for loading of the materials(ion gelation methods) was carried out weekly in the Postgraduate Laboratory of Physiology, Chemistry and Pharmacy Department/ College of Veterinary Medicine/ Al-Qasim Green University.

3.2.2.1 Preparing of chitosan nanoparticles(CNP)

The concentrations were prepared from a solution of chitosan provided by (Beijing) company according to the modulating method of Pires *et al*.,(2014), where the concentrations of 4 mg/ml of chitosan solution were prepared by adding (200 mg) of chitosan powder to (50ml) deionized distilled water (contains 1% acetic acid) for each and left for 24 hours at room temperature. Then, by continuous movement during stirring by a magnetic-bar in a hotplate stirrer for 30 minutes at 900 rpm, which leads to the formation of semi-colloidal solution. The pH was set at 4.6 by a pH meter by adding NaOH(0.1N), and exposure to sonication with a probe sonicator for 3 minutes, after which the solution was filtered with filter paper(400-800).

3.2.2.2 Preparation of TPP (Tripolyphosphate) solution

The TPP solution (Supplied by Daejung Chemicals and Metals Company) was prepared according to the method of Vaezifar *et al.,*(2013) by adding 250 mg of sodium tripolyphosphate powder to 100 ml of deionized distilled water to obtain a ratio of 0.25% W/V.

3.2.2.3 Loading of garlic extract on chitosan nanoparticles(GCNPs)

According to Ibrahim *et al.,*(2015) and Ali *et al.,*(2018), 200 mg of garlic powder was dissolved in 1 ml of distilled water and added by slow distillation to 50 ml of chitosan solution (4 mg/ml) to obtain a loading of garlic extract with chitosan under constant stirring in a hot plate stirrer for 30 minutes at 900 rpm. Then, the solution was exposed to sonication for 1 minute.

The solution was returned to continuous stirring. 10 ml of TPP (0.25%) was added with a ratio of 5:1 of solution by slow distillation and left to stirring for 30 minutes to allow of garlic extract particles to adsorb on the surface of the chitosan particles. Then the solution is returned to sonication for 1 minute, and then filtered with filter paper in order to get rid of non-bound particles. The solution was placed at a centrifugation force of 10,000 rpm for 15 minutes. The sediment was taken and the supernatant was disposed of. So we get garlic extract loaded on chitosan nanoparticles. The solution is kept at 4ºC.

3.2.3 Characterization of Nanoparticles

3.2.3.1 Particle size (PS) analysis

The particle sizes of different materials that was used in our study, unloaded and loading on chitosan nanoparticles, were measured using laser rays that penetrated the liquids containing the particles and determined the particle sizes as an average (0.5- 50000nm) within a specified period (90 second). The data is recorded in an electronic program prepared for this purpose that includes a curve graph and a table of particle size averages (Jabar, 2020). The test was conducted at the Nanotechnology and Advanced Materials Research Center / University of Technology.

3.2.3.2 Zeta potential analysis

The purpose of zeta potential analysis is to know the surface charge of the materials CNPs and G-CNPs to determine the validity of their use for movement within the body. The test was done by a zeta potential analyzer with a charge range of ($^{+}$150 - $^{-}$150mV) (Al-Saadi, 2020), and was conducted at the Center for Nanotechnology and Advanced Materials Research / University of Technology.

3.2.3.3 FTIR (Fourier transform infrared spectroscopy) analysis

To describe and define the active groups in materials prepared and garlic loaded on nanoparticles after the adsorption process, which gives the effectiveness of the effect inside the body related to reduction and stability, in addition to new chemical bonds of the materials needed for the colloidal solution, the absorption technique of the infrared spectrum was used. The solutions were exposed to centrifugation at 10000 rpm for 15 minutes, then the residue was washed with distilled water three times to remove unconnected particles suspended in the solution, after which it was dried at a temperature of 40^0C before being subjected and studied with a FTIR spectrophotometer (TENSOR 27,German).

The samples were mixed with a pure binder (KBr) and placed inside discs under high pressure. The test was done within the instrument wavelength of 400-4000 cm^{-1}, where there is spectroscopy containing a set of peaks reflecting the transmittance range of the infrared rays within certain frequencies representing the functional groups for each material (Tugarova *et al*, 2018; Al-Saadi, 2020). The test was conducted at the Center for Nanotechnology and Advanced Materials Research / University of Technology.

3.2.3.4 X-ray diffraction (XRD) analysis

The XRD measurement was carried out for the identification of the garlic extract loaded on chitosan nanoparticles(CNP-G).The biosynthesized CNP-G were freeze dried powdered in order to analyze XRD pattern .The phase formation and purity of metallic nanoparticles were checked through XRD patterns which were recorded using powder X-ray diffract meter. XRD analysis was performed using at a step size of 0.02˚, scanning rate of 2˚ in 2θ/min and a 2θ range from 30 C˚ to 80 C˚, a voltage of 40 kV and a current of 30 mA with Cu (Sadhasivam *et al*., 2010). The test was

conducted at the Center for Nanotechnology and Advanced Materials Research / University of Technology.

3.2.3.5 TEM (Transmission Electron Microscope)

Transmission electron microscopy analysis was used to determine the morphological and size properties of the nanoparticles, including the shape of the particles and their nanoscale size. In addition to the extent of isolation or accumulation of the particles with each other and the general appearance of their association after adsorption by the processes of nanoloading, where a single drop of the nanoscale solutions was placed on carbon-coated copper grids and left to dry over a period of time, the micrographs of the dry net were examined at 400 kV. Then the particle size, distribution, and shape were examined. The analysis was carried out at the Department of Nanotechnology of Medicine, Faculty of Advanced Technology of Medicine, University of Tehran, Iran (Lankalapalli *et al*., 2015).

3.2.4 *In vivo* experiment

3.2.4.1 The experimental animals

Sixty albino of male laboratory rats with weights ranging 250-300 g and 15 weeks of life were used in the experiment. The animals were placed in the animal house of the Biology department/ College of Science / University of Babylon, with environmental conditions that include moderate temperature, a 12 hour dark and 12 hour light cycle.

The animals were treated with the approval of the ethics committee at the department, where they were kept in meshed plastic cages containing sawdust; the pellets were fed (mix of corn, wheat and milk) and they drank tap water throughout the experiment. The animals were left to adapt for 14 days before starting the experiment for the period from February 2021 to April 2021.

3.2.4.2 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 (Gheith and El-Mahmoudy, 2018).Furthermore, complete blood count(CBC) were estimated to ensure the anemia was induced.

3.2.4.3 Determination of the doses

The effective dose for garlic extract and garlic loaded chitosan nanoparticles was determined as 34.5 mg/kg per day for 60 days orally according to the Al-Anawe (2020).

3.2.4.4 Dividing of experimental animals

Sixty albino of male rats was used. The animals were divided into 6 groups(10 rats for each group). 10 animals represented the control group, while 50 animals were divided into 5 groups. The animals were dosed orally from treatments by gavage to the stomach directly for a period of 60 days, according to following groups:

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

Group T1 : animals in this group were injected intraperitonial with 20mg/kg of phynelhydrazine to induced anemia as positive group.

Group T2 : animals in this group healthy were treated daily with 34.5 mg/kg of garlic extract.

Group T3 : animals in this group were injected with 20mg/kg of PHZ to induced anemia and treated daily with 34.5 mg/kg of garlic extract.

Group T4 : animals in this group healthy were treated daily with 34.5 mg/kg of CNP-G.

Group T5 : animals in this group were injected intraperitonial with 20mg/kg of phynelhydrazine to induced anemia and treated daily with 34.5 mg/kg of CNP-G.

3.2.4.5 Animals sacrifice

At the end of the experiment period (60 days), the animals were sacrificed after being anesthetized with xylazine(30 mg) and ketamine(mg). Then the blood samples (5ml) was collected after puncturing the heart with a 5ml syringe.

One ml was put into EDTA tubes for Complete Blood Count (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(-20ºC) until physio- biochemical analyses carried out which include electrolyte, total protein, albumin, erythropoietin, VB12, cyclooxygenase, hemopexin, total antioxidant capacity, and malondialdyhide and samples from bone were taken for histological study.

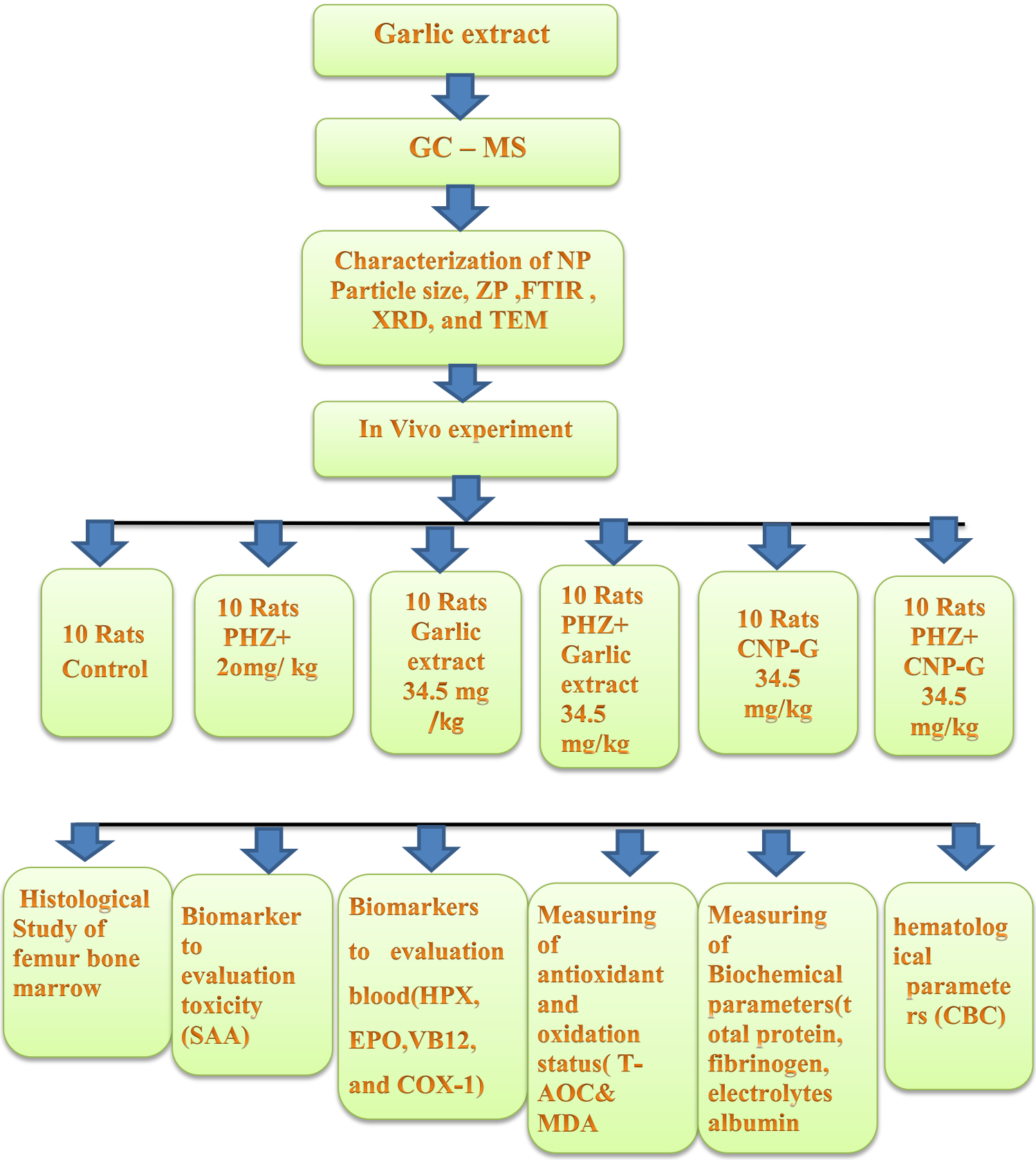

Figure 3-1: Experimental design

3.2.5 Hematological studies

All of hematological profile (CBC) have been done by using 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 at 20 µl volume is taken by probe, and taken out of the device, after a minute the result was appeared.

3.2.6 Measuring of Biochemical parameters

3.2.6.1 Total serum protein, Albumin, and Electrolytes (Na^+, K^+, Ca^{++}, Iron)

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

3.2.6.2 Fibrinogen plasma

1. add 10 µl serum with 190 µl buffer in test tube (Hitach cup).
2. put 150 µl of above solution in 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 (China).

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

3.2.7.1 Procedure

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 (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 T-AOC and MDA standards was added to standard wells. Note: antibody don't added to standard well because the standard solution contains biotinylated antibody.
4. Forty µl sample was added to sample wells and then added 10µl anti-VB12 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 60min. 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 T-AOC, and MDA 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 T-AOC, and MDA Stop solution was add to all wells. The blue color will changed 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.

3.2.7.2 Calculations of concentration

A standards curve were plotted for the absorbance versus the concentration of the standards. To calculate each sample concentration of T-AOC and MDA , 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 T-AOC and MDA as shown in figures (3-2 and 3-3) respectively.

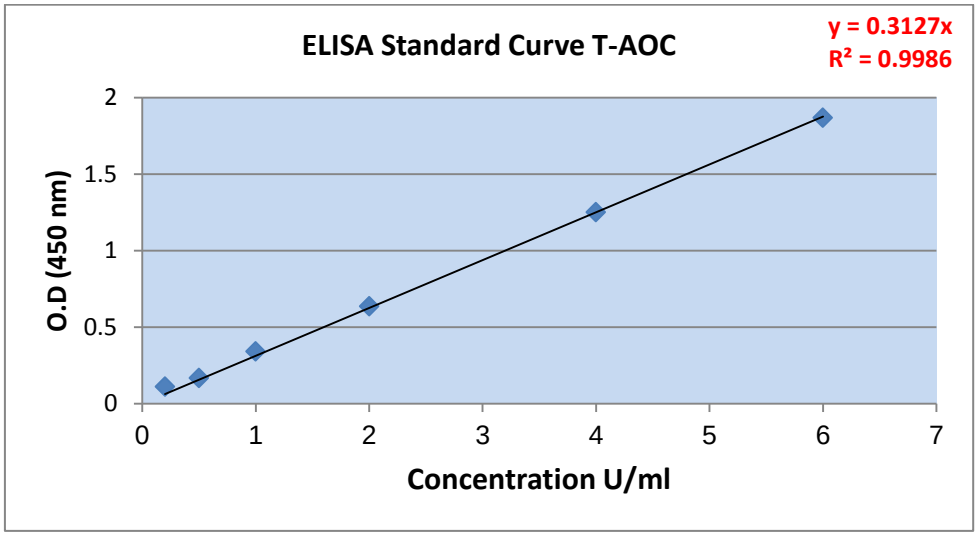

Figure 3-2: Standard curve for determination of TAO-C concentration.

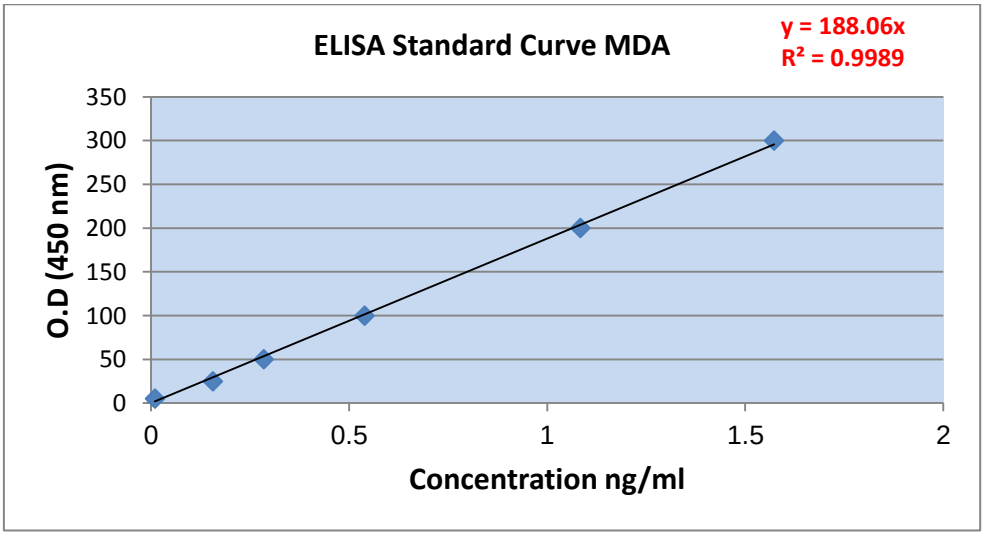

Figure 3-3: Standard curve for determination of MDA concentration.

3.2.8 Biomarkers to evaluation blood (HPX, EPO,VB12, and COX-1)

3.2.8.1 Procedure

The kits were used in this assay include Rat hemopexin (HPX), Rat erythropoietin (EPO), Rat vitamin B12 (VB12), and Rat cyclooxygenase (COX-1) with cat.No. (E1084Ra , E0293Ra ,E0610Ra, and E1245Ra) 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 HPX, EPO, VB12 , and COX-1 standards was added to standard wells. Note: antibody don't added to standard well because the standard solution contains biotinylated antibody.
4. Forty µl sample was added to sample wells and then added 10µl anti-VB12 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 60min. 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 HPX, EPO, VB12 , and COX-1 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 HPX, EPO, VB12 , and COX-1 Stop solution was add to all wells. The blue color will changed into yellow immediatly.
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.

3.2.8.2 Calculations of concentration

A standards curve were plotted for the absorbance versus the concentration of the standards. To calculate each sample concentration of HPX, EPO, VB12 , and COX-1 , 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 HPX, EPO, VB12 , and COX-1 as shown in figures (3-4, 3-5, 3-6 and 3-7) respectively.

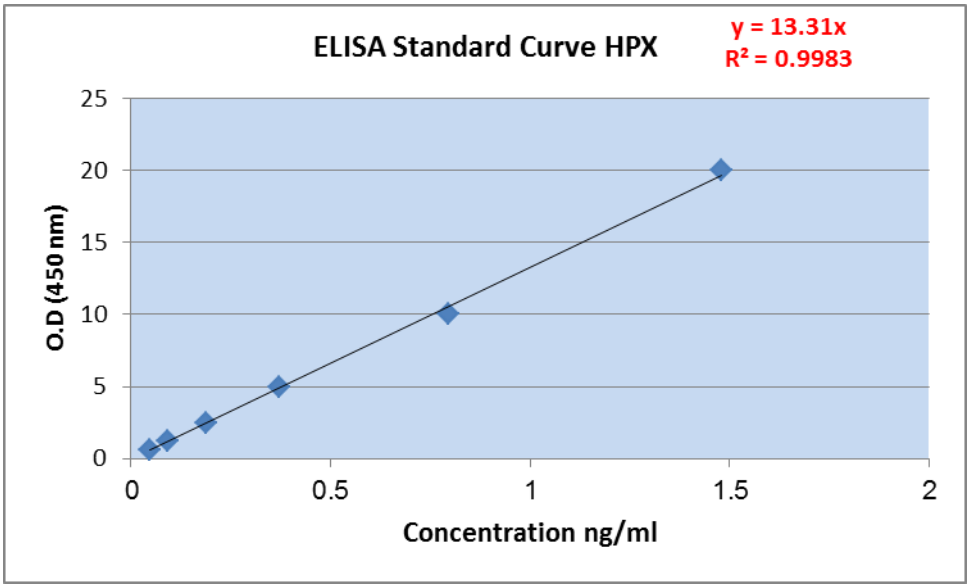

Figure 3-4: Standard curve for determination of HPX concentration.

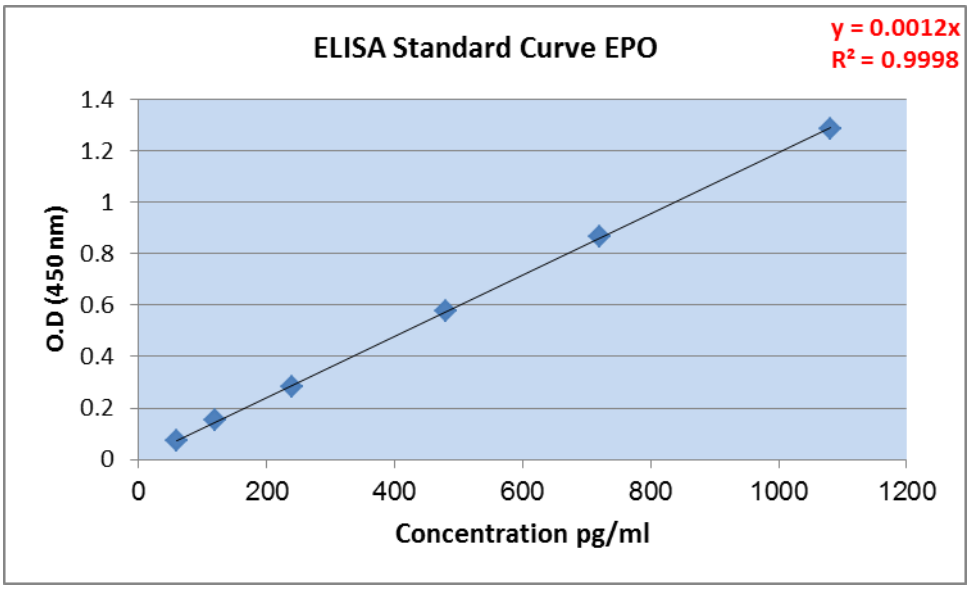

Figure 3-5: Standard curve for determination of EPO concentration.

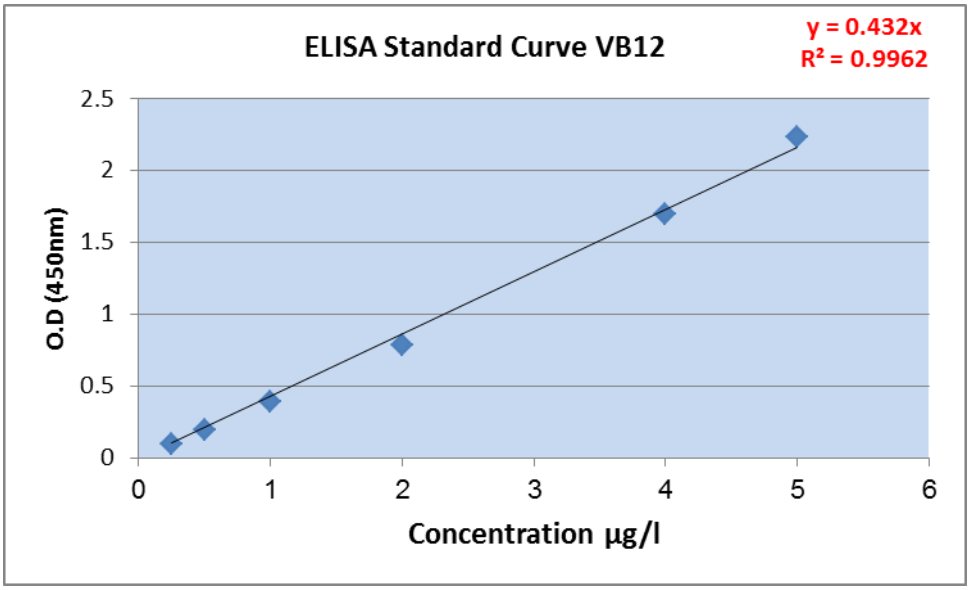

Figure 3-6: Standard curve for determination of VB12 concentration.

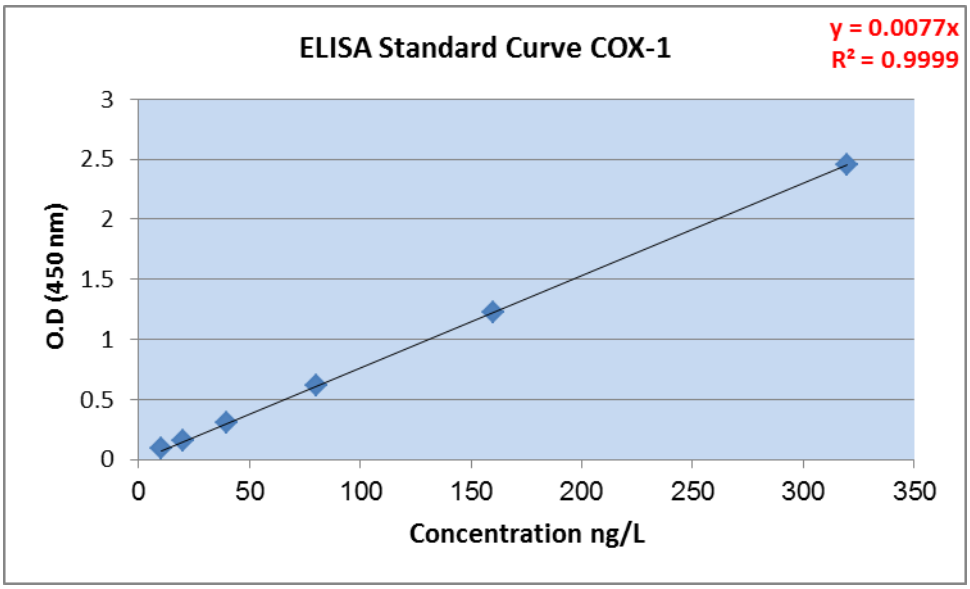

Figure 3-7: Standard curve for determination of COX-1 concentration.

3.2.9 Biomarker to evaluation toxicity (serum amyloid SAA)

3.2.9.1 Procedure

The kit were used in this assay include Rat Serum amyloid A (SAA) with Cat. No. (E-EL-R3026), using ELISA kit by method of Sandwich-ELISA were determined according to (Jordan ,2005) as following **:**

1. One hundred uL of SAA standard working solution were added to the first two columns in duplicating to one well each side by side. Then 100uL of sample was added to others wells. The plate were cover with plate sealer, incubated for 90 min. at 37ºC.
2. The liquid of each well were removed out, did not washed. 100µL of biotinylated detection Ab working solution added to each well.
3. The solution was aspirated from each well, of washing buffer(350uL) added to each well. Soaking(1-2 min) and the solution aspirated from each wall and pat it dry against clean absorbent paper. This wash step repeated 3 times.
4. One hundred µL of HRP-conjugate working solution was added to each well, covered with the plate sealer. Incubated for 30min. at 37ºC.
5. The solution was aspirated from each well, the wash process repeated for five times as conducted in step3.
6. 90µL of substrate reagent added to each well. Covered with a new plate sealer. Incubated for about 15min. at 37ºC.
7. 50 µL of stop solution of each SAA added to each well.
8. The optical density (OD value) of each well determined at once with a micro-plate reader set to 450nm.

3.2.9.2 Calculations of concentration:

The standards curve were plotted for the absorbance versus the concentration of the standards. To calculate each sample concentration of SAA, 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 SAA, as shown in figure (3-8).

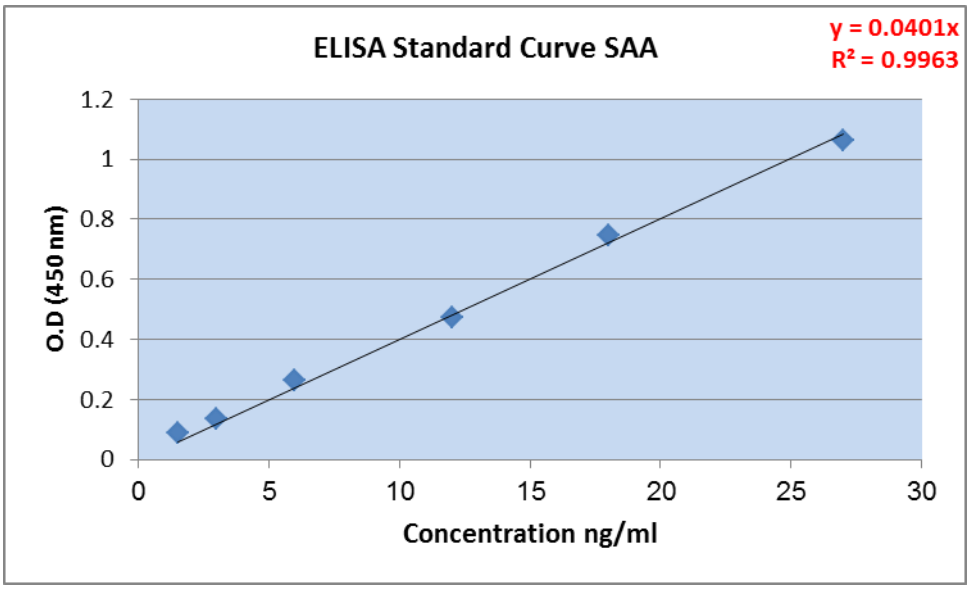

Figure 3-8: Standard curve for determination of SAA concentration.

3.2.10 Histological study

3.2.10.1 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.

3.2.10.2 Preparation of femoral bone sample

Preparation of femoral bone sample as according of(Luna,1968)

1. Femoral bone samples were collected and fixed in 10% formalin for 48 hours.
2. 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.
3. 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).
4. The bone tissue samples were then trimmed and sectioned at 4μm thickness using semiautomatic microtome (Histo-Line MRS3500, Italy).
5. 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).

6. 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.
7. 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.
8. 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.
9. 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.
10. Bone tissue sections rinsed in 95% alcohol for 20 seconds and counterstained with eosin stains for 5 minute.
11. 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.
12. Tissue section were observed using a light microscope at 40,100, 200, 400 and 1000 magnifications.

3.2.11 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 ± SE and statistical significance was accepted at $P \leq 0.05$ (Al-Ukaelii and Al-Shaeb, 1998).

4. Results

4.1 GC-MS analysis for garlic extract

Analysis of the components of garlic in ethanol and methanol solvent was performed by the (GC-MS) technique (Gas Chromatography- Mass Spectrometry).The total ion chromatogram (TIC) (Figure 4-1) for this method appeared as separate peaks of the components according to their abundance against the retention time (Rt).

According to the results of the analysis by (GC-MS) technique of garlic, there were (20)chemical compounds that appeared depending on the retention time (Table 4-1), where the Allyl disulfide compounds appeared in an area of 66.70% of the content, the most common of which was Diallyl disulfide with the highest area among the compounds reaching (10.01%) and a retention time of 7.854, followed by Disulfide, methyl 2-propenyl at 8.87% with a retention time of 4.661, whereas 3-Vinyl-1,2-dithiacyclohex, appeared with a percent 3.88% and a retention time of 9.439, 6,6-Dimethyl-2 methylenebicyclo [3.1.1]heptane with a retention time of 4.926 and area is 3.00%, Methoxy acetic acid (2.44%), 2,2-Dimthyl-3-methylenbicyclo [2.2.1]heptane (2.34%). The result also raveled that there were many compounds with small area included in the extract, such as 6,6-Dimethyl-2-(3-oxobutyl)bicycle[3.1.2]heptan-3-one with retention time 6.658 and area 0.83%, 2-propanol,1-methoxy that was appeared at a percentage of 0.29% with a retention time of 5.033, 1,6-Heptadiene, 2-methyl was found at a retention time of 5.839 with 0.10%, also Cyclobutane,1,2-dipropenyl (0.11%), o-Allylhydroxylamine (0.46%), Hexane,3-methoxy (0.48%), o-Allylhydroxylamine (0.11%), and 3-Vinyl-1,2-dithiacyclohex (0.15%).

It is worth noting, in the GC-MS analysis in this study, the emergence of five compounds not previously proven within the chemical composition of the garlic extract, namely (Carbamaldehyde, 1-propen, 1-bromo, 2-

Ethylpyrrole, Methacrylic anhydride, and 2-propyne nitrile,3), thus adding new compounds within the components of this plant that may be have different physiological and medical roles.

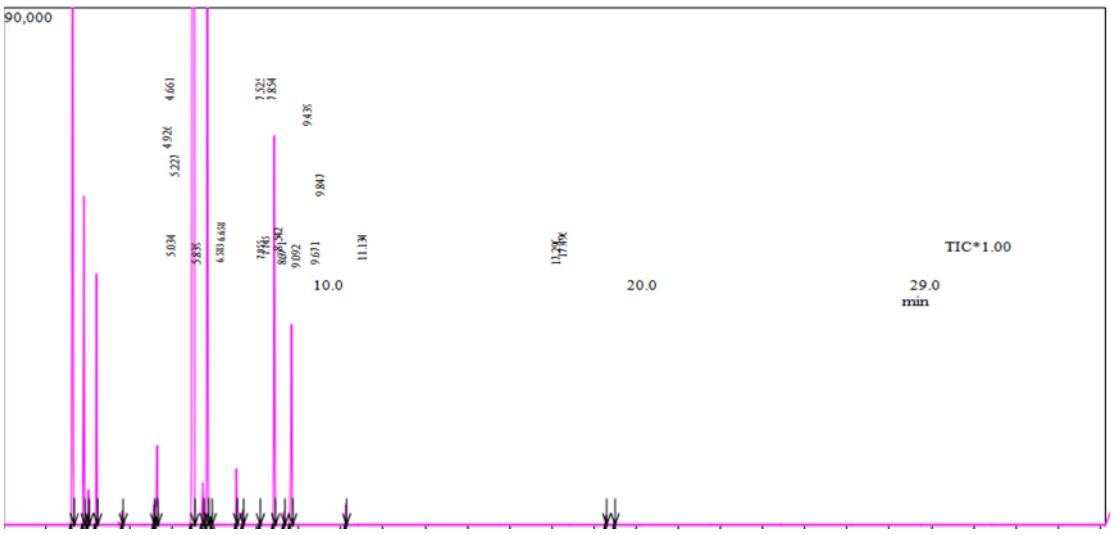

Figure 4-1: Chromatogram Garlic H 1 C:\GCMSsolution\Sample\Garlic H 1.QGD

Table 4-1:Chemical compounds of garlic extract by using GC-MS analysis according to the retention time.

Peak No	Compounds name	R-Time	C.Area %	M.W	Formula
1	Disulfide, methyl 2-propenyl	4.661	8.87	120	$C_4H_8S_2$
2	6,6-Dimethyl-2-methylenebicyclo[3.1.1]heptane	4.926	3.00	136	$C_{10}H_{16}$
3	2-Propanol,1-methoxy	5.033	0.29	90	$C_4H_{10}O_2$
4	2,2-Dimethyl-3-methylenebicyclo[2.2.1]heptane	5.227	2.34	136	$C_{10}H_{16}$
5	1,6-Heptadiene, 2-methyl	5.839	0.10	110	C_8H_{14}
6	Cyclobutane,1,2-dipropenyl	6.583	0.11	136	$C_{10}H_{16}$
7	6,6-Dimethyl-2-(3-oxobutyl)bicycle[3.1.1]heptan-3-one	6.658	0.83	208	$C_{13}H_{20}O_2$
8	Allyl disulfide	7.525	66.70	146	$C_6H_{10}S_2$
9	o-Allylhydroxylamine	7.745	0.46	73	C_3H_7NO
10	Diallyl disulfide	7.854	10.01	146	$C_6H_{10}S_2$
11	**Carbamaldehyde**	7.955	0.02	45	CH_3NO
12	Hexane, 3-methoxy	8.542	0.48	116	$C_7H_{16}O$
13	**1-Propen, 1-bromo**	8.691	0.08	120	C_3H_5Br
14	**2- Ethylpyrrole**	9.092	0.03	95	C_6H_9N
15	3-Vinyl-1,2-dithiacyclohex	9.439	3.88	144	$C_6H_8S_2$
16	o-Allylhydroxylamine	9.671	0.11	73	C_3H_7NO
17	Methoxy acetic acide	9.847	2.44	174	$C_8H_{14}O_4$
18	3-Vinyl-1,2-dithiacyclohex	11.134	0.15	144	$C_6H_8S_2$
19	**Methacrylic anhydride**	17.290	0.08	154	$C_8H_{10}O_3$
20	**2-Propynenitrile,3**	17.496	0.02	69	C_3FN

4.2 Characterization of Nanoparticles

4.2.1 Particle size (PS) analysis

The particle size was measured by laser rays (Particle size 90Plus, USA) that penetrated the liquid containing the molecules of garlic extract revealed that the average size of these particles before loading was 11.6 nm within the particle size range (10.3-13 nm), whereas showed that the average particle size of garlic extract after loading on chitosan nanoparticles (CNP-G) was an average of 25.9 nm within the range of particle sizes (12.7-52.7 nm) (Fig.4-2).

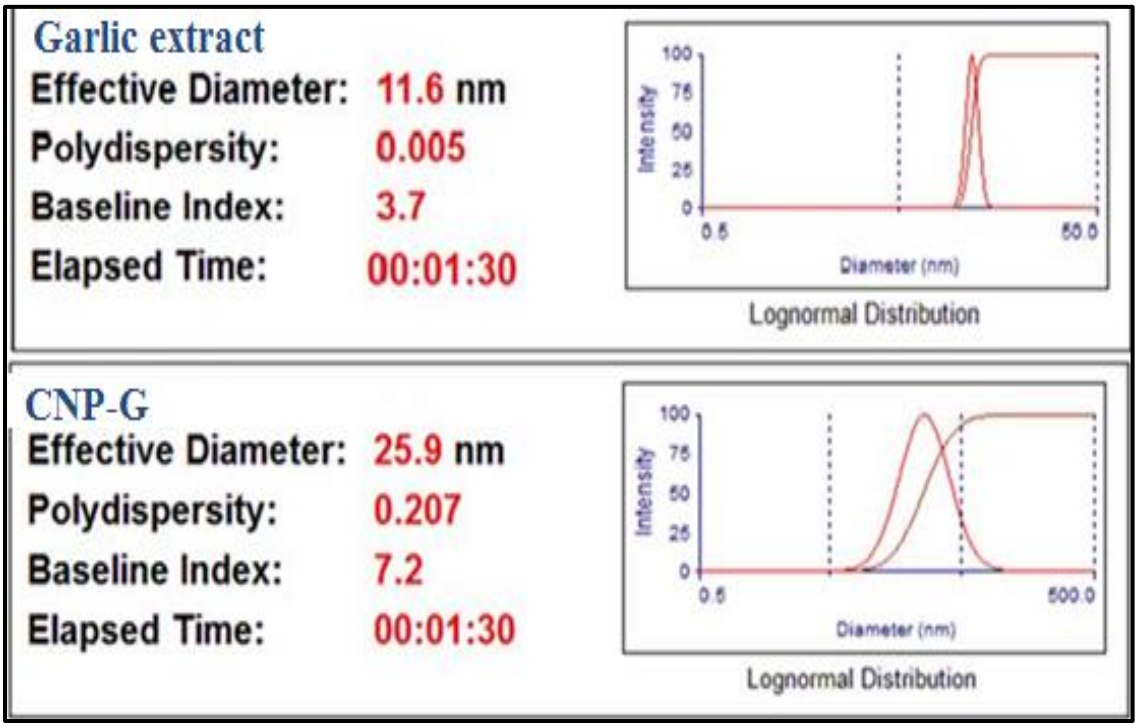

Figure 4-2: particle size PS analysis of garlic extract and CNP-G

4.2.2 Zeta potential analysis

Measuring the zeta potential includes knowing the surface charge of particles and the substances adsorbed on their surface. The Zeta potential curve in the current study measured by using a Zeta potential analyzer (Zeta plus, USA) exhibited the highest surface charge of chitosan particles in aqueous solution was $^{+}$30.04mV, while the Zeta potential of Tripolyphosphate (TPP) showed the highest value of $^{-}$19.62mV, which encourages strong

bonding with chitosan according to the charge difference between them (figure 4-3and figure 4-4).

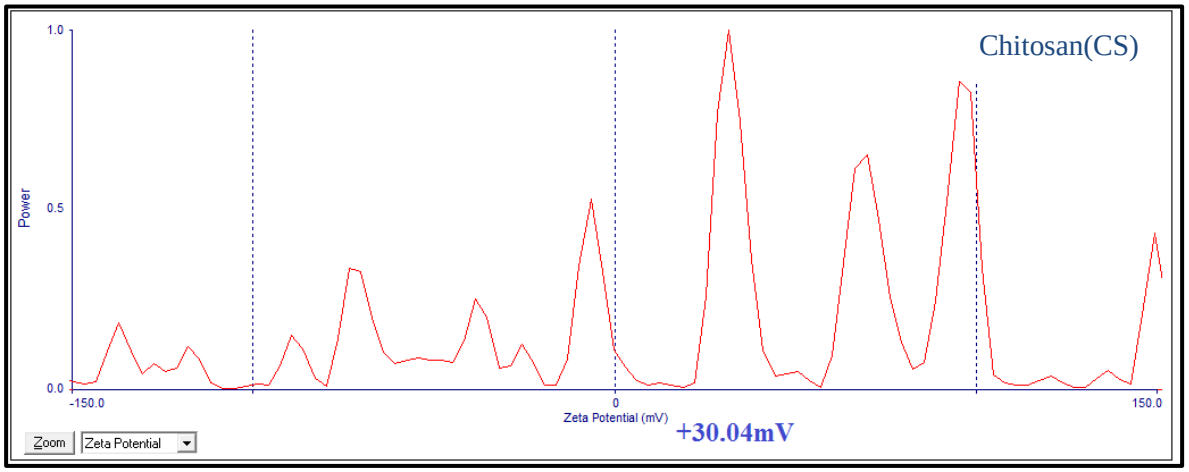

Figure 4-3: Zeta potential of chitosan nanoparticles (CNP)

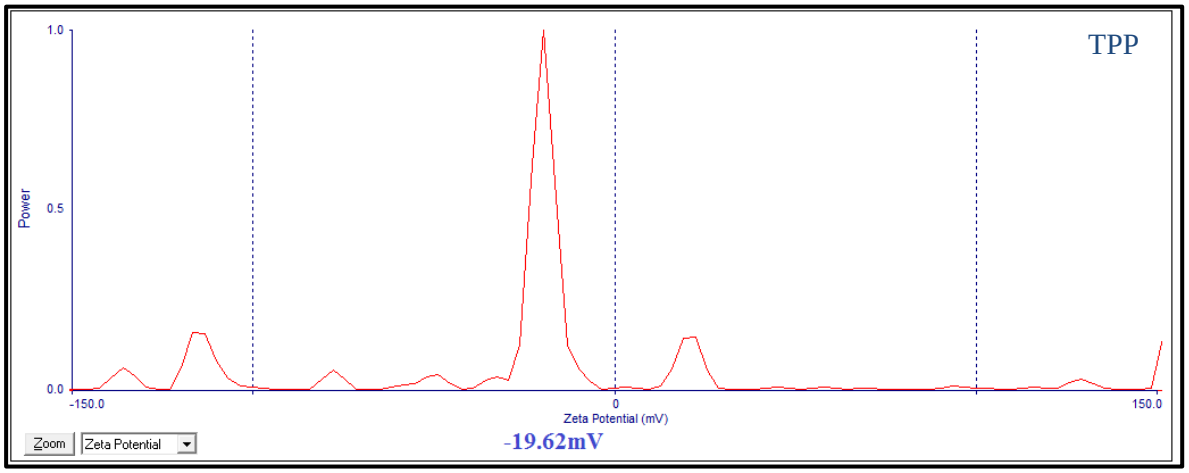

Figure 4-4: Zeta potential of tripoly phosphate (TPP).

As for the zeta potential curve of the garlic extract particles in aqueous solution, the highest potential of the surface was $^{+}$28.80mV (figure 4-5), while loading of garlic extract molecules in the colloidal solution of chitosan (CNP-G) showed zeta potential increased in the direction of positivity to reach $^{+}$48.43mV, confirming stable positive charge after attachment of the extract molecules to the surface of the chitosan particles, which gives more stability to the solution (Figure 4-6).

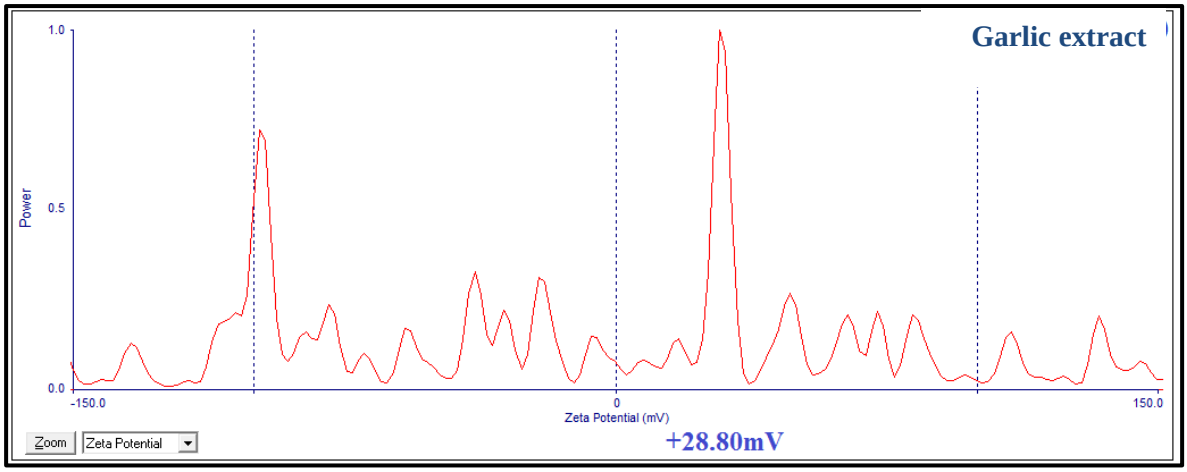

Figure 4-5: Zeta potential of garlic extract

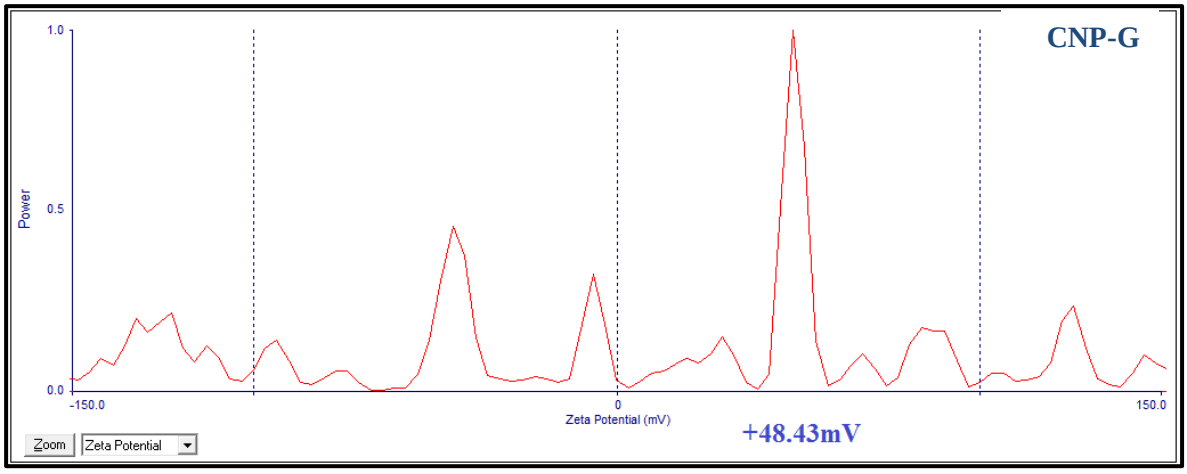

Figure 4-6: Zeta potential of CNP-G.

4.2.3 FTIR (Fourier transform infrared spectroscopy) analysis

The transmittance curves of the infrared frequencies of materials used in the study exhibited the presence of many different peaks representing the functional groups for each material according to the absorption capacity of the IR frequencies.

The infrared spectrum of chitosan-TPP solution figure (4-7) indicates the presence of several peaks located at many wavenumbers. The peak at 896.9 cm-1 may be attributed to the bending vibration of the CH aliphatic bond. The peak at 1653 cm^{-1} is due to the stretched vibration of the C=C bond. The peak at the wavelength of 1035.77 cm-1 is attributed to the stretching vibration for C=O. The peaks 1082.07, 1155.36 and 1259.52 cm^{-1} may be attributed to the stretching of C-O bond that is due to the alcohol

groups in the polymer. The peaks 1379.10, 2873.94, 3851.85, 3917.43 and 3417.86 cm-1 are due to the stretching vibration of the OH group in the alcohol groups, while 3417.86, 3429.43 and 2873.94 cm-1 are the result of the stretching of the bonds of the amine group NH.

The structure of the CNP and CNP-G in figure (4-8) were confirmed by FTIR, where the spectrum of the CNP shows more broad absorption bands at 3358.07 cm^{-1}. This broad band might be corresponded to hydroxyl groups (OH) stretching vibrations of water molecules, OHs and NH_2 stretching vibrations of free amino groups. The two bands observed at 2927.94 and 2879.72 cm^{-1} correspond to asymmetric stretching of CH_3 and CH_2 in both CNPs and CNP-G. The observed band at 2221 cm^{-1} is attributed to C–N group of C–NH_2. In addition, the stretching band of C–O in chitosan spectrum was observed at 1038.48 cm^{-1} in all types of NPs.

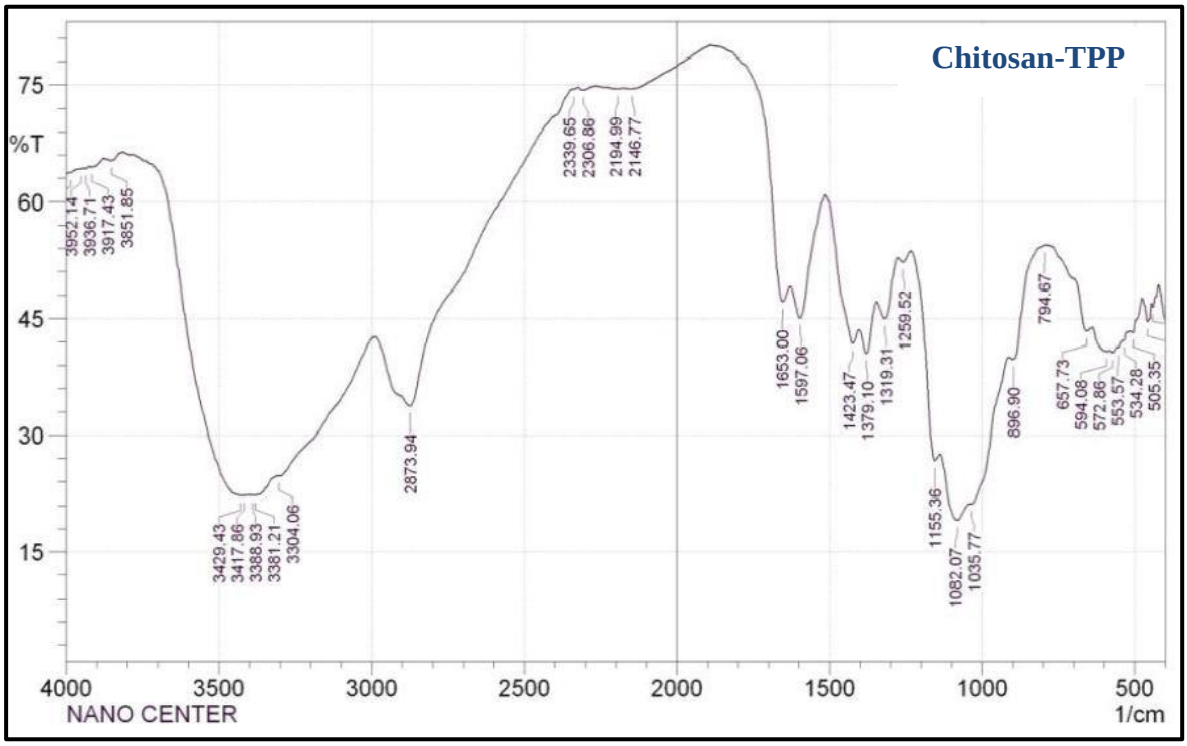

Figure 4-7: FTIR spectroscopy of Chitosan-TPP solution.

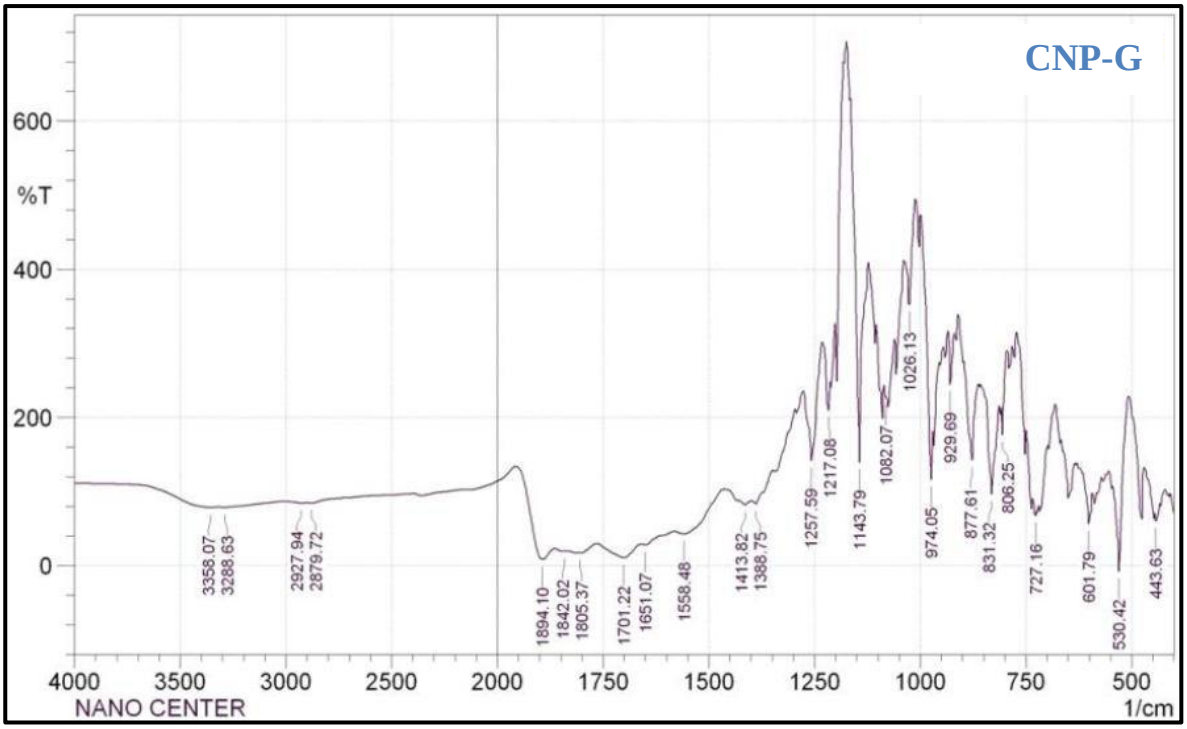

Figure 4-8: FTIR spectroscopy of CNP-G.

4.2.4 X-ray diffraction (XRD) analysis

The structural nature (crystal and phase purity) of the prepared CNP-G was studied using the X-ray diffraction XRD patterns. Figure (4-9) , represents the XRD analysis of CNP-G .

Size of nanoparticle was calculated using Debye–Scherrer equation that suggesting the spherical in shape crystal structure of the nanoparticle.

D =$K\lambda$ / $\beta cos\theta$, D- particle size in nm, K is the constant is Sherrer (0.9), λ X-ray wavelength, β- FWHM, θ- Reflective angle of Bragg.

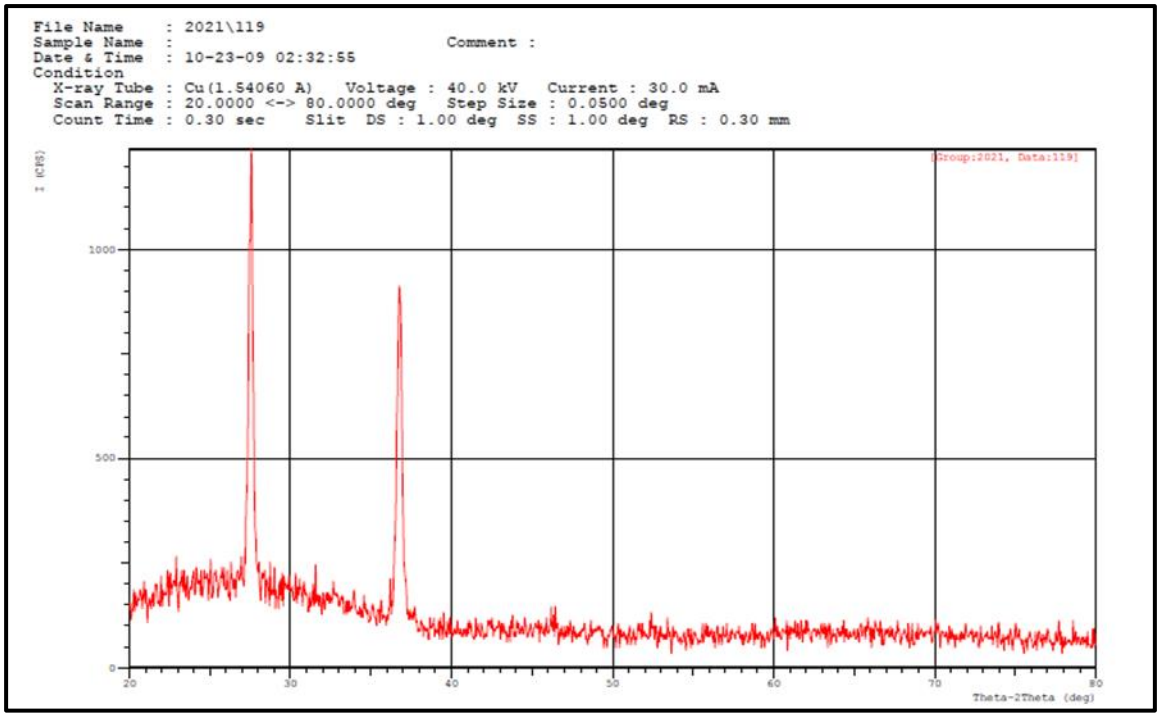

Figure 4-9: X-ray diffraction (XRD) of CNP-G.

4.2.5 TEM (Transmission electron microscope) analysis

TEM image of CNP-G confirmed the shape and distribution after conjugation between polymer nanoparticles and garlic extract molecules through the two magnification forces, where the shape of the particles was observed changing from round or spherical to irregular with rough surfaces representing the adsorbed material on the surface of the polymer, as well as high dispersion was observed of particles in the colloidal solution, which confirms the homogeneity of the solution and the success of the loading process, and this was previously observed during the decrease of polydispersity in particle size analysis figure (4-10).

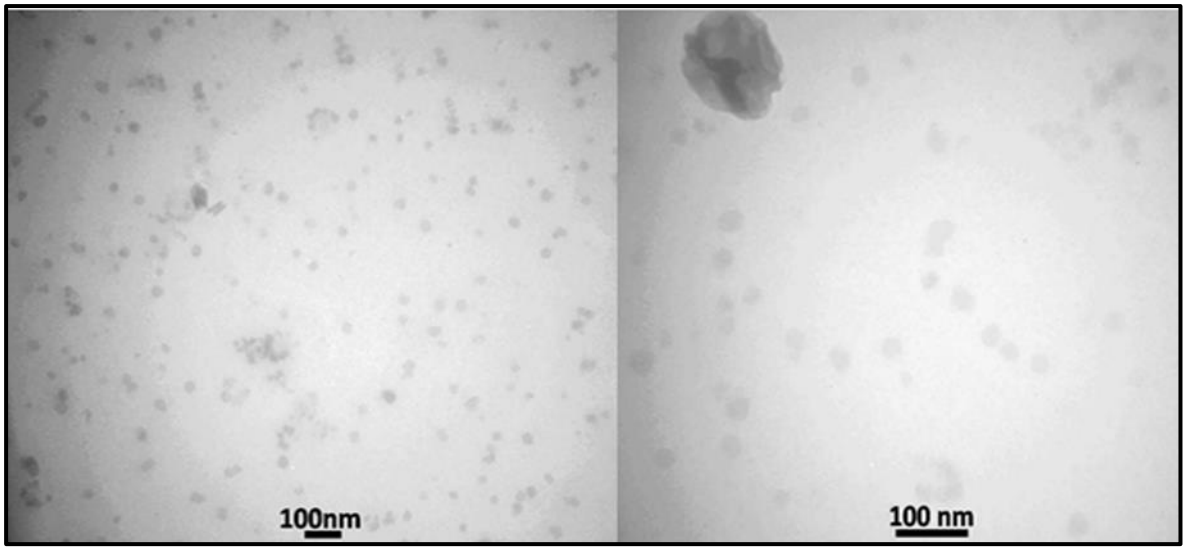

Figure 4-10:Transmission electron micrograph (TEM) image for CNP-G.

4.3 *In vivo* experiment

4.3.1 Hematological studies

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

The present study of RBC_s data table (4-2), showed a significant increase ($p < 0.05$) in (T2,T4, and T5) compared with anemic group and control group and there is non a significant ($p > 0.05$) difference between them, on the other hand blood samples of RBC_S showed prominent reduction in T1 group that received PHZ when compared with control and among treated groups. In addition to the our data showed a significant decrease in Hb and PCV of anemic group, on the other aspect the data of Hb and PCV in treatment groups appear a significant increase($p < 0.05$) compare with anemic group .

Table 4-2:Effect of garlic extract and garlic loaded chitosan nanoparticles on some hematological parameters of anemic male rats.

Groups of experiment	$RBC_S \times 10^6/mm^3$	Hb g/dl	PCV %
NC	**6.05 ± 0.27 c**	**12.06 ± 0.60 b**	**41 ± 0.39 a**
T1	**3.43 ± 0.051 d**	**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 b**	**36.1 ± 0.58 b**
T4	**7.92 ± 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**

- The value represent mean ± S E
- 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 garlic loaded chitosan nanoparticles, and **T5** : anemic group received garlic loaded chitosan nanoparticles.

4.3.1.2 Red blood corpuscles indices

The mean of (MCV, MCH, and MCHC) in anemic group showed a significant increase ($P < 0.05$) as compared with NC, while treatment groups (T4 and T5) that received CNP-G at dose 34.5 mg/kg of MCH and MCHC recorded a significant decrease when compared with anemic group and negative control group and T4 of MCV showed non significant difference when compared with NC but T5 recorded a significant decrease as compared with NC .

Table 4-3: Effect of garlic extract and garlic loaded chitosan nanoparticles on RBC$_s$ indices of anemic male rats.

Groups of experiment	MCV µm^3	MCH Pg	MCHC g/dl
NC	53.9 ± 0.40 b	18.9 ± 0.27 a	35.5 ± 0.86 b
T1	56.3 ± 1.48 a	19.8 ± 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 b	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

- 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 garlic loaded chitosan nanoparticles, and **T5** : anemic group received garlic loaded chitosan nanoparticles.

4.3.1.3 Platelets count and ESR

The result of platelets count showed significant decrease ($P < 0.05$) in (T2, T3, T4, and T5) compared with NC and T1. While T2 group recorded non significant variation ($P < 0.05$) when compared with NC group.

The ESR were decreased significantly ($P < 0.05$) in T2,T3,T4, and T5 compared to T1, while non significant between NC, T2, T3, and T5. Also there were a significant decrease ($P < 0.05$) in T4 and T5 compared to T2 and T3.

Table 4-4: Effect of garlic extract and garlic loaded chitosan nanoparticles on Platelet count and ESR of anemic male rats.

Groups of experiment	Platelets mm^3	ESR mm/h
NC	**727 ± 7.8 b**	**2.8 ± 0.16 b**
T1	**984 ± 9.2 a**	**3.34 ± 0.28 a**
T2	**673 ± 28 bc**	**2.60 ± 0.16 b**
T3	**568 ± 5 c**	**2.53 ± 0.16 b**
T4	**554 ± 3 c**	**2.32 ± 0.16 c**
T5	**622 ± 2 c**	**2.3 ± 0.16 bc**

- 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 garlic loaded chitosan nanoparticles , and **T5**: anemic group received garlic loaded chitosan nanoparticles.

4.3.1.4 Total and differential leukocytes

In the present study, the data in the (table 4-5) confirmed significant ($P < 0.05$) elevation in leukocyte and monocyte, granulocyte, and lymphocyte in group that received PHZ to induce anemia when compared with negative control and treatment groups. While the treatment group (T3 anemic group that received garlic extract) of lymphocyte showed non significant ($P > 0.05$) deference as compared with anemic group.

Anemic group that orally received garlic loaded chitosan nanoparticles (T5) showed a significant decrease ($P < 0.05$) in leukocyte , lymphocyte, and monocyte as compared with anemic group and negative control group.

Table 4-5: Effect of garlic extract and garlic loaded chitosan nanoparticles on Total and differential leukocytes of anemic male rats.

Groups of experiment	WBC×10^3/mm^3	Monocyte%	Granulocyte %	Lymphocyte%
NC	**9.67 ± 0.53 c**	**9.21 ± 0.16 b**	**10.66 ± 0.91 b**	**74.7 ± 2.1 d**
T1	**12.3 ± 0.60 a**	**11.7 ± 0.80 a**	**17.85 ± 1.71 a**	**83.4 ± 0.71 a**
T2	**9.54 ± 0.59 c**	**8.12 ± 0.81 bc**	**9.85 ± 1.26 b**	**80.7 ± 1.52 b**
T3	**11.98 ± 0.59 b**	**7.7 ± 0.33 c**	**9.51 ± 0.82 b**	**83.5 ± 1.05 a**
T4	**9.56 ± 0.32 c**	**7.7 ± 0.21 c**	**10.34 ± 0.41 b**	**71.4 ± 2.24 e**
T5	**9.30 ± 0.52 c**	**7.60 ± 0.31 c**	**9.64 ± 1.20 b**	**79.9 ± 1.45 c**

- 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 garlic loaded chitosan nanoparticles, and **T5** : anemic group received garlic loaded chitosan nanoparticles.

4.3.2 Measuring of Biochemical parameters

4.3.2.1 Total serum proteins, albumin, and fibrinogen

The data in(table 4-6), confirmed that serum total protein concentration in the treatment groups showed clear significant elevation ($P < 0.05$) as compared with anemic group. Although the (T3, T4, and T5) recorded non significant ($P > 0.05$) variation between them.

Albumin (g/L) concentration in group that injection by PHZ recorded significant ($P < 0.05$) reduction when compared with control and all others treated groups. On other hands (T3 and T4) showed non significant ($P > 0.05$) difference between them. Conversely, the mean difference of fibrinogen showed a significant ($P < 0.05$) increase in anemic group as compared with negative control group and decrease in all treatment groups when compared with anemic group.

Table 4-6: Effect of garlic extract and garlic loaded chitosan nanoparticles on total protein, albumin, and fibrinogen of anemic male rats.

Groups of experiment	Total protein g/dl	Albumin g/dl	Fibrinogen g/L
NC	**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**

- 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.

4.3.2.2 Electrolytes of blood (Na^+, K^+, Ca^{++}, Iron)

The present study confirmed that there was a significant ($P < 0.05$) increase in mean values of serum K^+ concentration in T1 and T5 group as compared with NC, while T4 recorded non significant deference with NC group. Serum sodium and Calcium concentration in anemic group recorded a significant ($P \leq 0.05$) decrease as compared with all others groups. While the treatment groups T2 and T5 showed a significant increase($P < 0.05$) as compared with other treatment groups and anemic group.

While iron in T1revealed significant ($P < 0.05$) increase as compared with NC group and all treatment groups, and all treatment groups that received garlic extract and CNP-G recorded a non significant deference when compared with NC group.

Table 4-7: Effect of garlic extract and garlic loaded chitosan nanoparticles on blood electrolytes of anemic male rats.

Groups of experiment	Potassium mmol/L	Sodium mmol /L	Calcium mg/dl	Iron mg/dl
NC	**7.36 ± 0.57 c**	**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 b**	**145.4 ± 11.6 a**	**11.69 ± 0.17 a**	**166.3 ± 10.5 c**
T3	**8.58 ± 0.20 b**	**143.7 ± 12.5 b**	**11.21 ± 0.11 ab**	**169.5 ± 3.2 c**
T4	**6.64 ± 0.10 c**	**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**

- 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 garlic loaded chitosan nanoparticles , and **T5** : anemic group received CNP-G.

4.3.3 Measuring of antioxidant and oxidation status

4.3.3.1 Total antioxidant capacity (T-AOC)

Our data in figure (4-11) appear a significant ($P < 0.05$) increase of total antioxidant capacity in all groups treatment that received garlic extract and CNP-G, and increase in T4 and T5 that received CNP-G when compared with anemic group and showed non significant ($P > 0.05$) differences between T2 , T3 and negative control group.

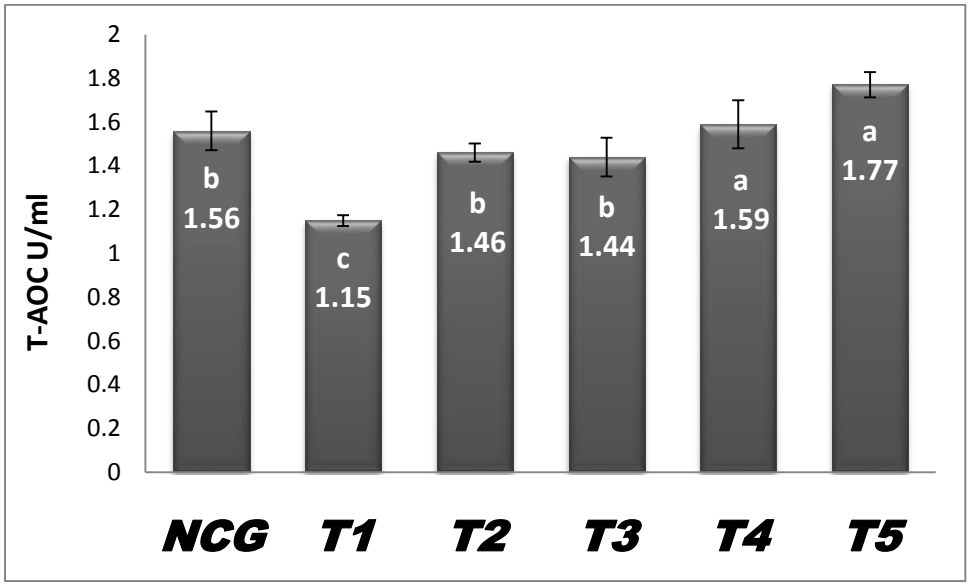

Figure 4-11: Effect of garlic extract and garlic loaded chitosan nanoparticles on total antioxidant capacity (T-AOC) level

- 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 garlic loaded chitosan nanoparticles, and **T5** : anemic group received garlic loaded chitosan nanoparticles.

4.3.3.2 Malondialdehyde (MDA)

The present study illustrated in figure (4-12) showed a significant increase ($P < 0.05$) in serum MDA of anemic group as compared with negative control and all treatment groups, T5 (that received CNP-G at dose 43.5mg/kg) recorded decreased in mean value of MDA, T3 showed non significant with NCG and T2 and T4 showed non significant between them.

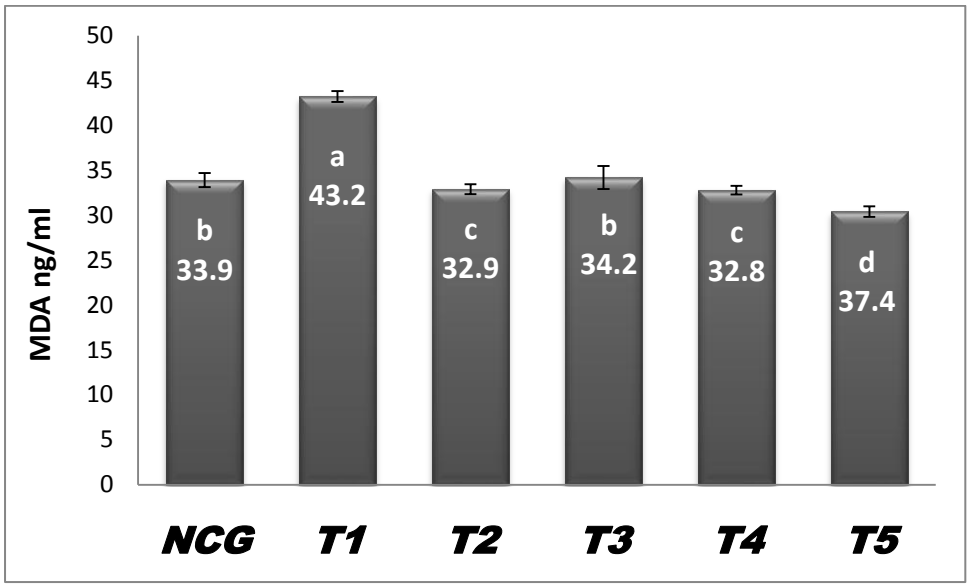

Figure 4-12: Effect of garlic extract and garlic loaded chitosan nanoparticles on malondialdehyde (MDA) level

- 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 garlic loaded chitosan nanoparticles, and **T5** : anemic group received garlic loaded chitosan nanoparticles.

4.3.4 Biomarkers to evaluation blood (HPX, EPO, VB12, and COX-1)

4.3.4.1 Hemopexin (HPX)

The present study in figure (4-13) showed there was a significant ($p > 0.05$) decrease in anemic group of serum HPX when compared with negative control group and all treatment groups, while T5 (that received CNP-G at dose 34.5 mg/kg) recorded a significant ($p < 0.05$) increase when compared with anemic and control groups.

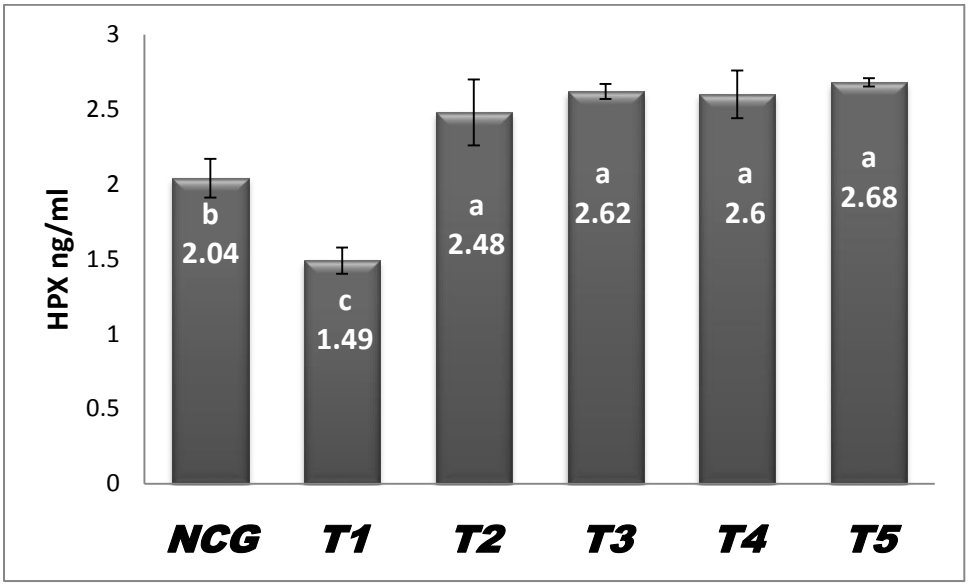

Figure 4-13: Effect of garlic extract and garlic loaded chitosan nanoparticles on hemopexin (HPX) level

- 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 garlic loaded chitosan nanoparticles, and **T5** : anemic group received garlic loaded chitosan nanoparticles.

4.3.4.2 Erythropoietin (EPO)

Our data in figure (4-14) confirmed high serum concentration of EPO in positive control group that received PHZ at dose 20 mg/kg while all others treatment groups recorded non significant ($p > 0.05$) variation as compared with negative control group and between them .

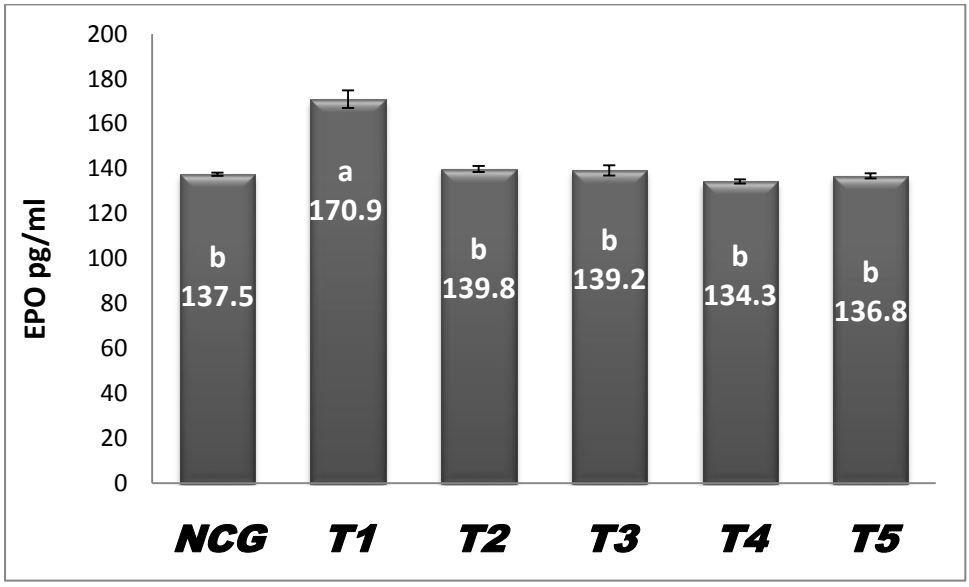

Figure 4-14: Effect of garlic extract and garlic loaded chitosan nanoparticles on erythropoietin (EPO) level

- 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 garlic loaded chitosan nanoparticles, and **T5** : anemic group received garlic loaded chitosan nanoparticles.

4.3.4.3 Vitamin B12 (VB12)

Our data in figure (4-15) confirmed high VB12 in serum of treated group that received garlic loaded chitosan nanoparticles as compared with anemic group , while T2, T3, and T4 reported non significant ($p > 0.05$) differences as compared with negative control group.

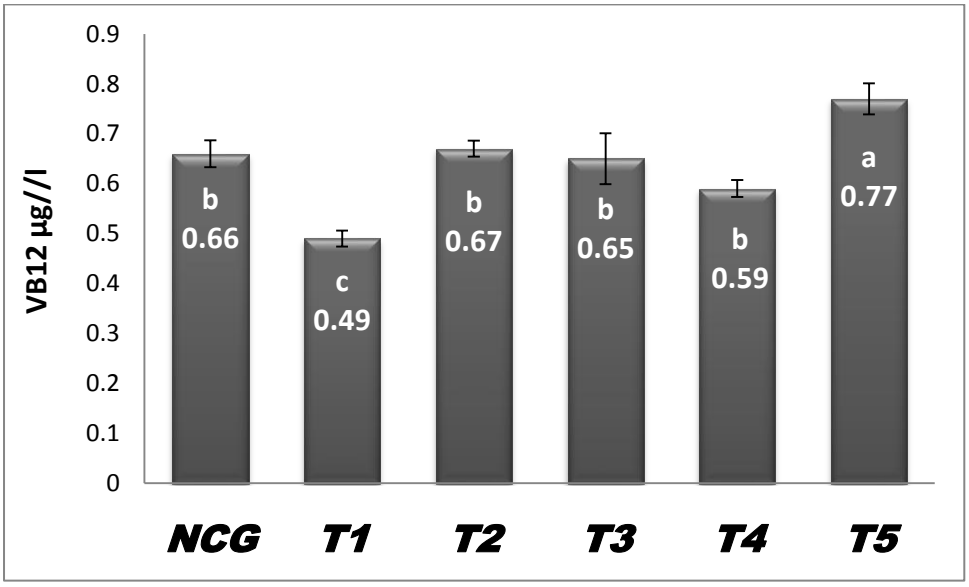

Figure 4-15: Effect of garlic extract and garlic loaded chitosan nanoparticles on vitamin B12 (VB12) level

- 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 garlic loaded chitosan nanoparticles, and **T5** : anemic group received garlic loaded chitosan nanoparticles.

4.3.4.4 Cyclooxygenase (COX-1)

The present study reveal that there was a significant ($p < 0.05$) elevation in cyclooxygenase in anemia group as compared with control group and all others treatment groups. While recorded significant decrease ($p > 0.05$) in T5 that treatment with CNP-G as compared with CNG.

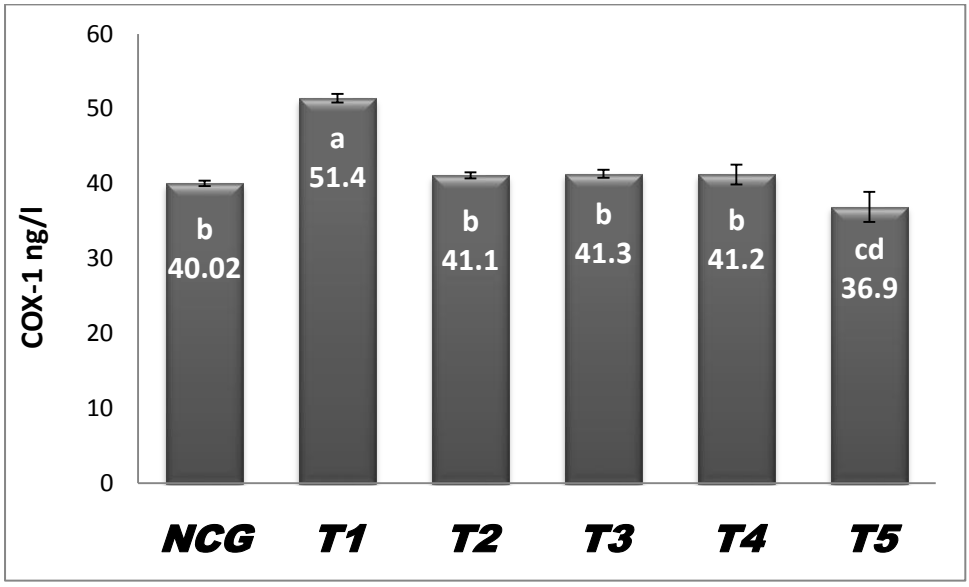

Figure 4-16: Effect of garlic extract and garlic loaded chitosan nanoparticles on cyclooxygenase (COX-1)

- 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 garlic loaded chitosan nanoparticles, and **T5** : anemic group received garlic loaded chitosan nanoparticles.

4.3.5 Biomarker to evaluation toxicity

4.3.5.1 Serum amyloid A (SAA)

This study showed a significant reduction ($p > 0.05$) of serum SAA in all treatment groups and negative control group when compared with anemic group, and T4 and T5 (that received CNP-G) recorded a significant decrease as compared with T2 and T3(that received garlic extract) and control group.

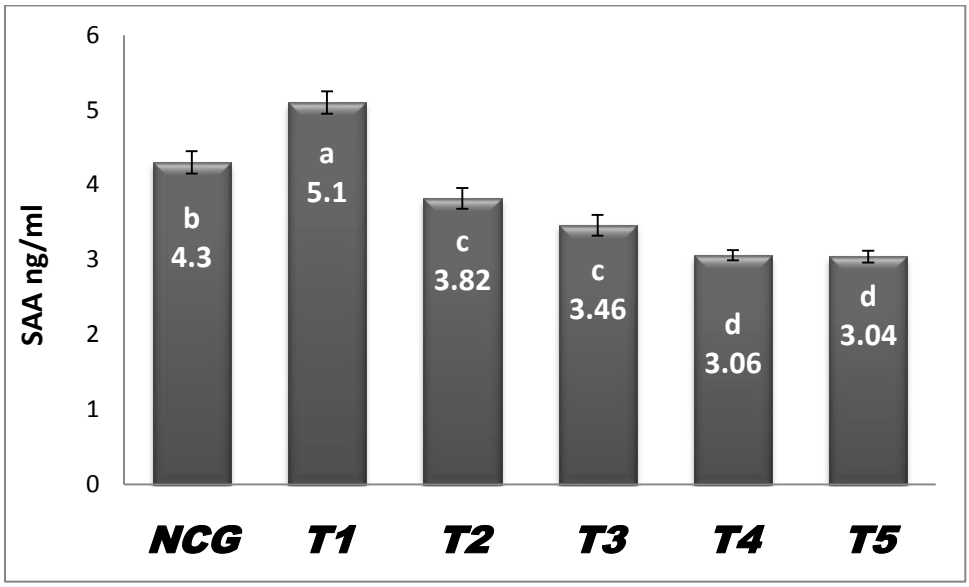

Figure 4-17: Effect of garlic extract and garlic loaded chitosan nanoparticles on serum amyloid (SAA) level

- 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 garlic loaded chitosan nanoparticles , and **T5** : anemic group received garlic loaded chitosan nanoparticles.

4.3.6 Histological study

4.3.6.1 Histological evaluation of femur bone marrow

Examination of femur bone marrow of anemia-induced rats figure 4-19 A and B, showed the severe hypoplasia of erythroid cells was observed, where the low density of erythroid cells formed huge spaces within the bone marrow tissue, also failure of bone marrow to produced erythrocyte as compared with negative control and T2 groups respectively in figure 4-18 and figure 4-20,that showed the normal architecture of bone marrow.

Anemic rats group(T3) in figure 4-21 that received garlic extract at does 34.5 mg/kg showed the mild hypoplasia of erythroid cells, where the decrement in erythroid cells replaced with fatty cells that observed as vacuoles within the bone marrow tissue, but when received garlic extract loaded chitosan nanoparticles (CNP-G)(T5) in figure 4-23 showed normal architecture of bone morrow with improvement in cells proliferation mitotic activity was also seen with presences of megakaryocytic cells.

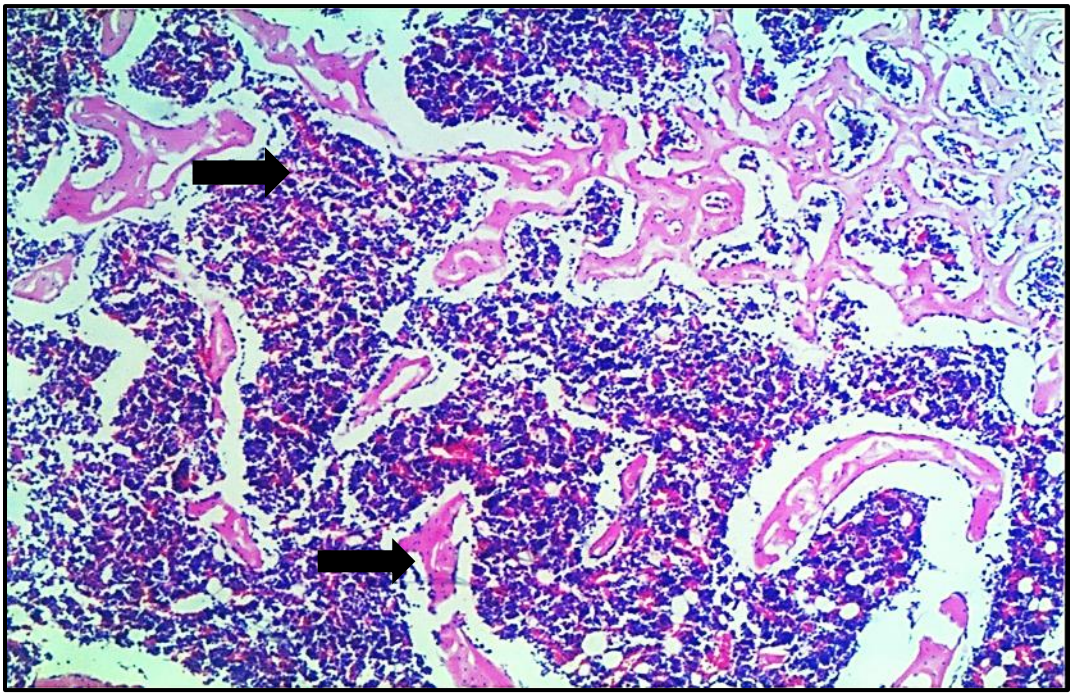

Figure 4-18: Histological section of femur bone marrow of rat for negative control group shown normal architecture of bone morrow, the bone marrow occupy all the space between bone trabeculae tissue (black arrows). H&E stain , x40.

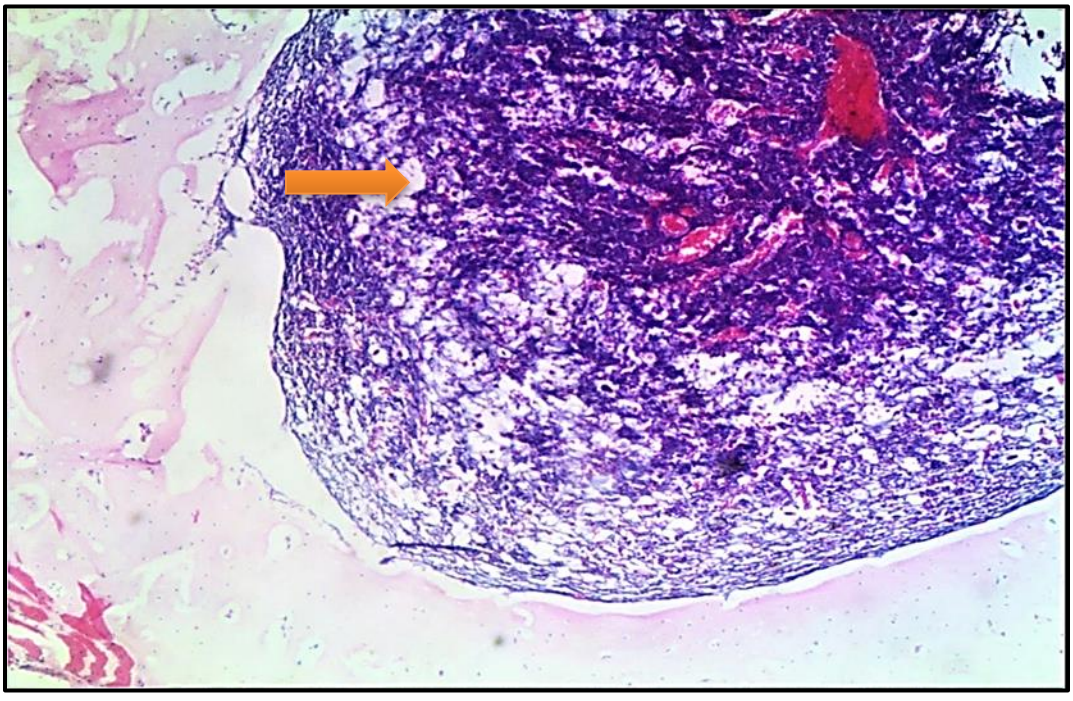

Figure 4-19 A: Histological section of femur bone marrow of rat for positive control group (T1) shown severe hypoplasia of erythroid cells was observed, where the low density of erythroid cells formed huge spaces (orange arrow) within the bone marrow tissue H&E stain , x40.

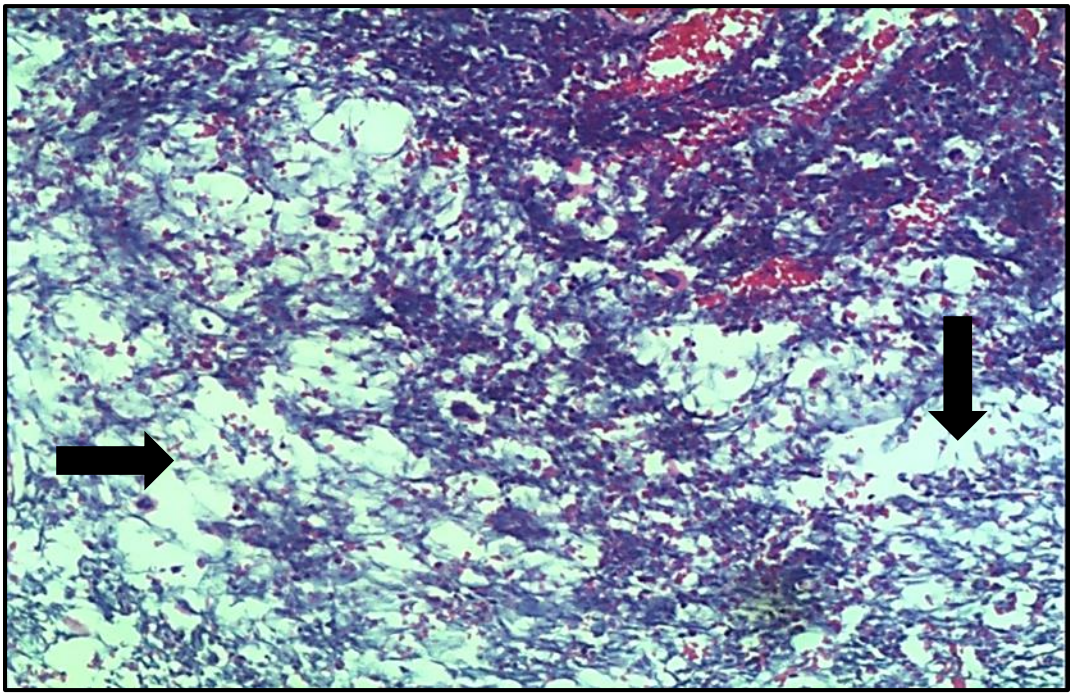

Figure 4-19 B: High magnification histological section of femur bone marrow of rat for positive control group (T1) shown failure of bone marrow to produce erythrocytes. Characterized by presences of multiple spaces within bone marrow (black arrow) H&E stain , x100.

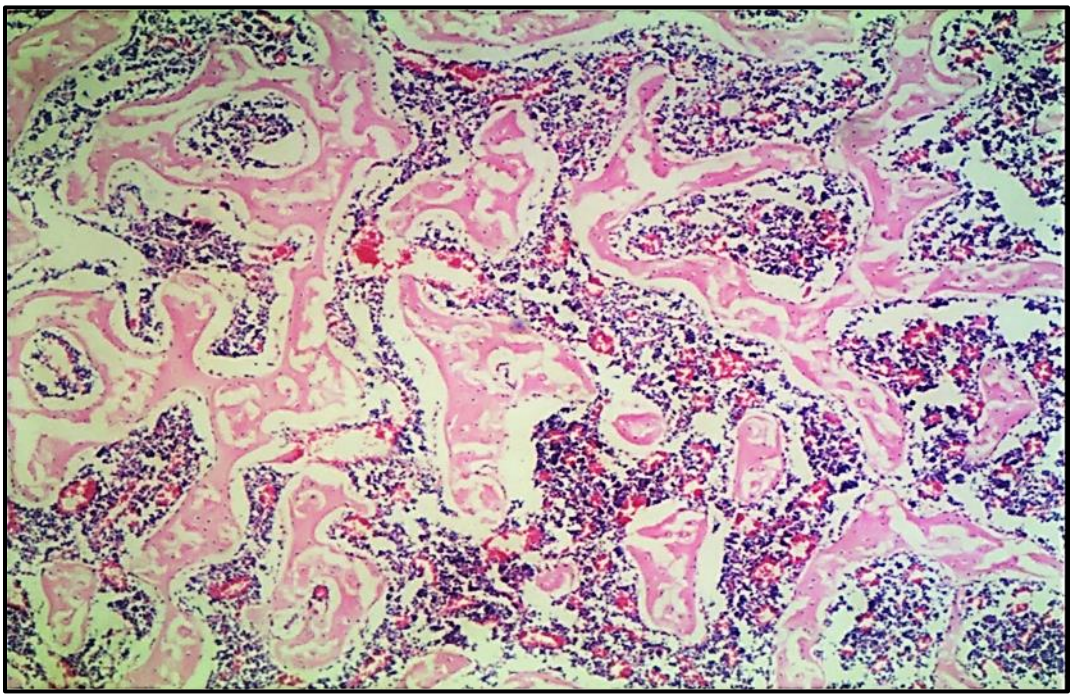

Figure 4-20: Histological section of femur bone marrow of rat for treatment group (T2) shown normal histological architectures H&E stain , x40.

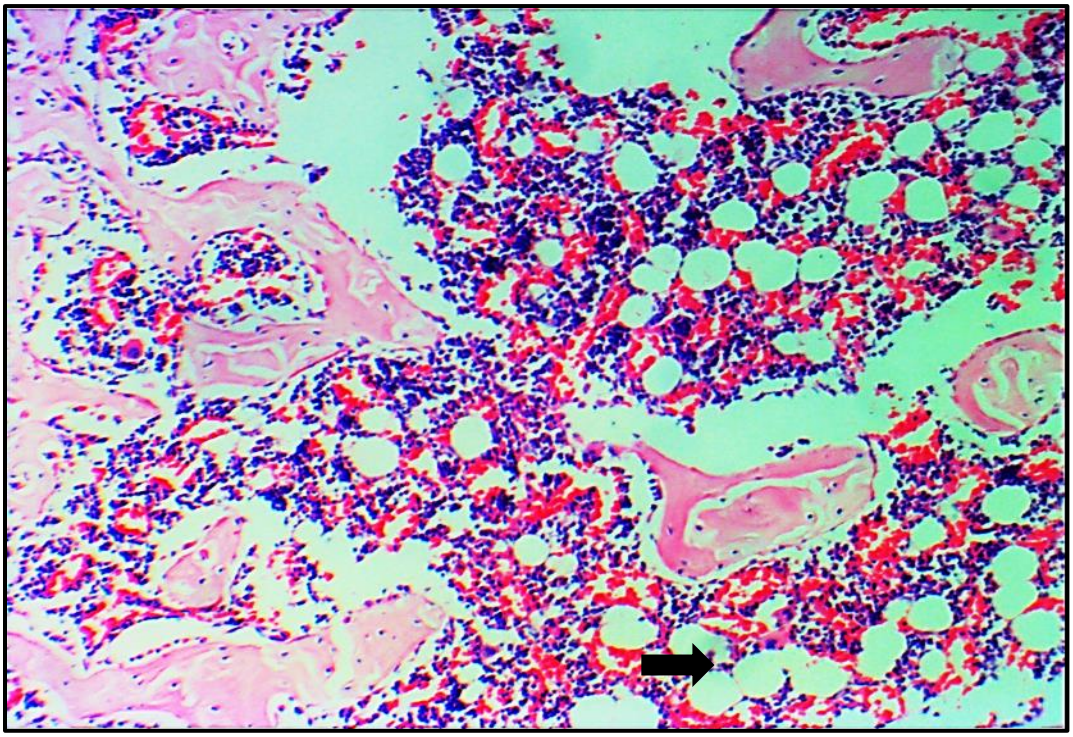

Figure 4-21 A: Histological section of femur bone marrow of rat for treatment group (T3) shown mild hypoplasia of erythroid cells ,where the decrement in erythroid cells replaced with fatty cells that observed as vacuoles (black arrows) within the bone marrow tissue H&E stain , x400.

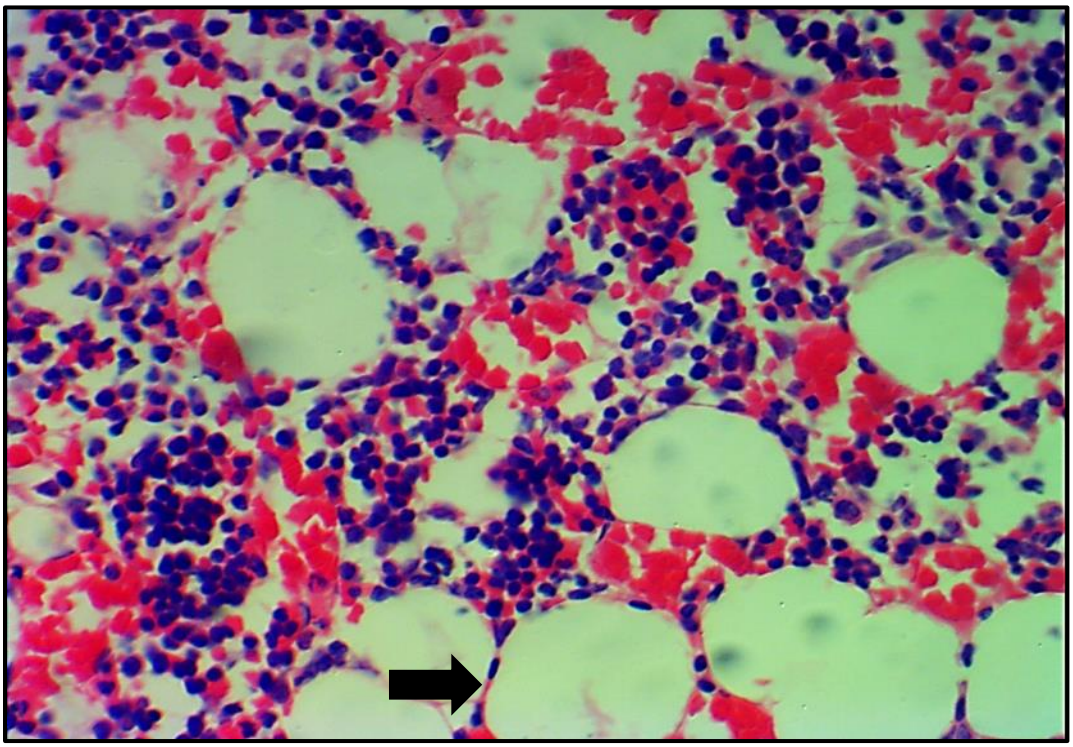

Figure 4-21 B: High magnification histological section of femur bone marrow of rat for treatment group (T3) shown mild hypoplasia of erythroid cells indicated the hypo-proliferative bone marrow (black arrows). H&E stain x1000.

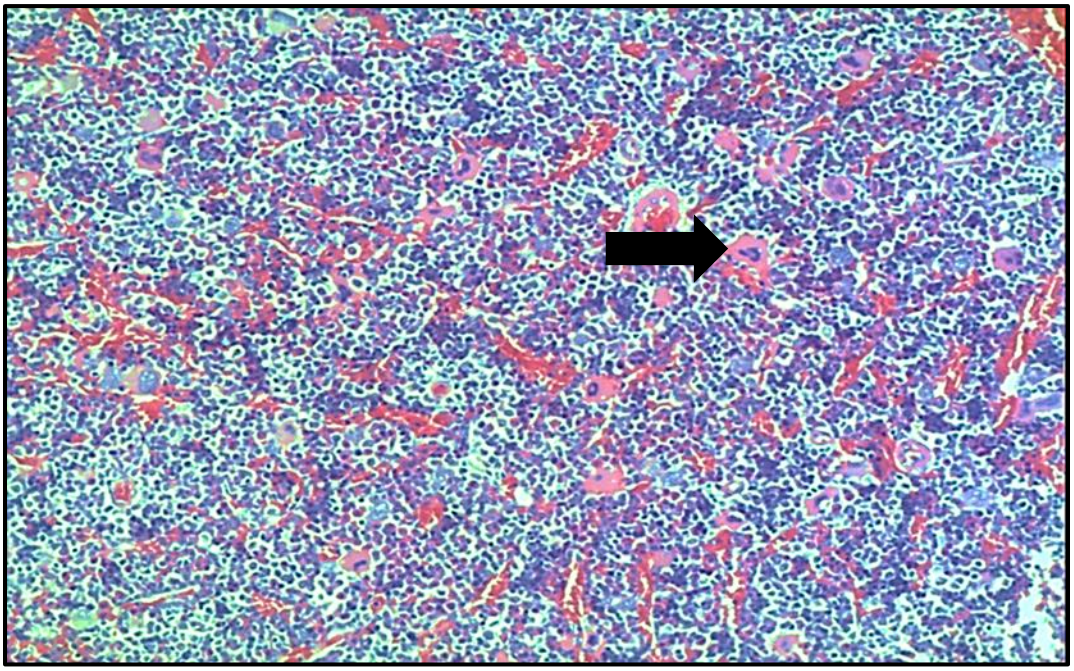

Figure 4-22 A: Histological section of femur bone marrow of rat for treatment group (T4) shown hyperplasia of the bone marrow cells with presences of megakaryocytic cells (black arrow). H&E. stain .x100.

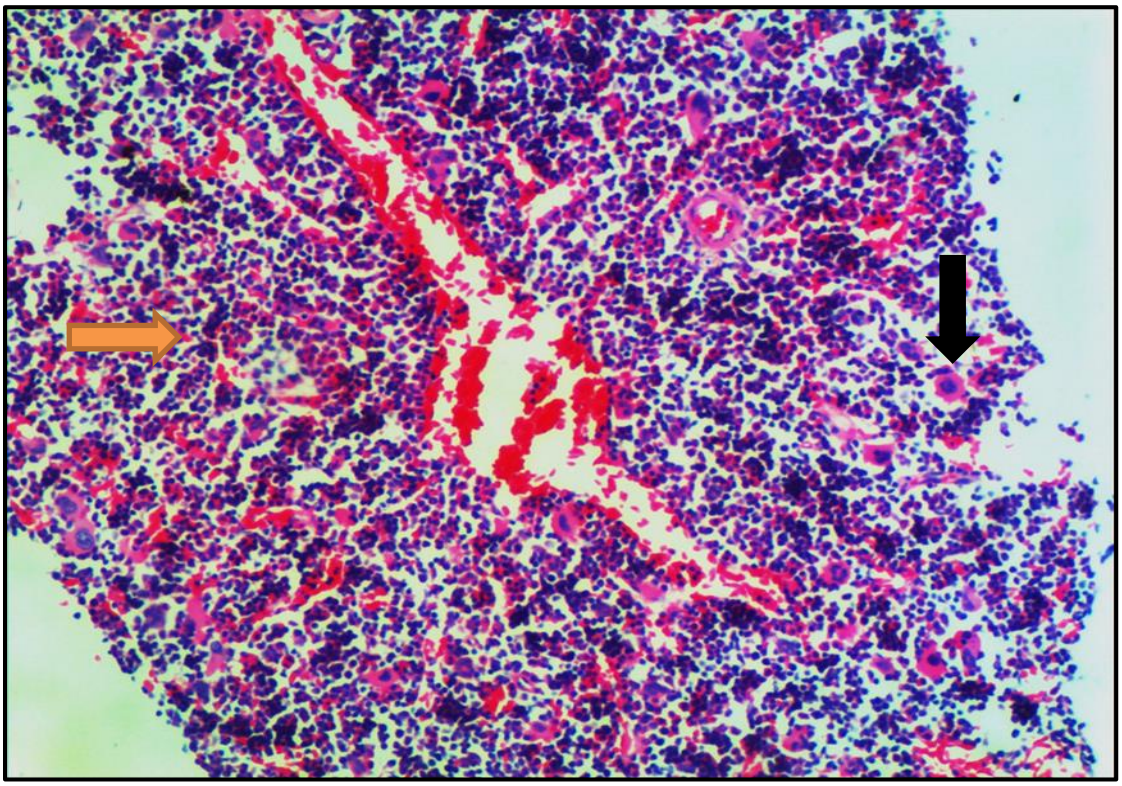

Figure 4-22 B:High magnification histological section of bone marrow of rat femur bone marrow for treatment group (T4) shown mild bone morrow hyperplasia , indicating the healthy proliferation activity of bone marrow (orange arrows) was also observed with presences of megakaryocytic cells (black arrow). H&E, stain. 200x.

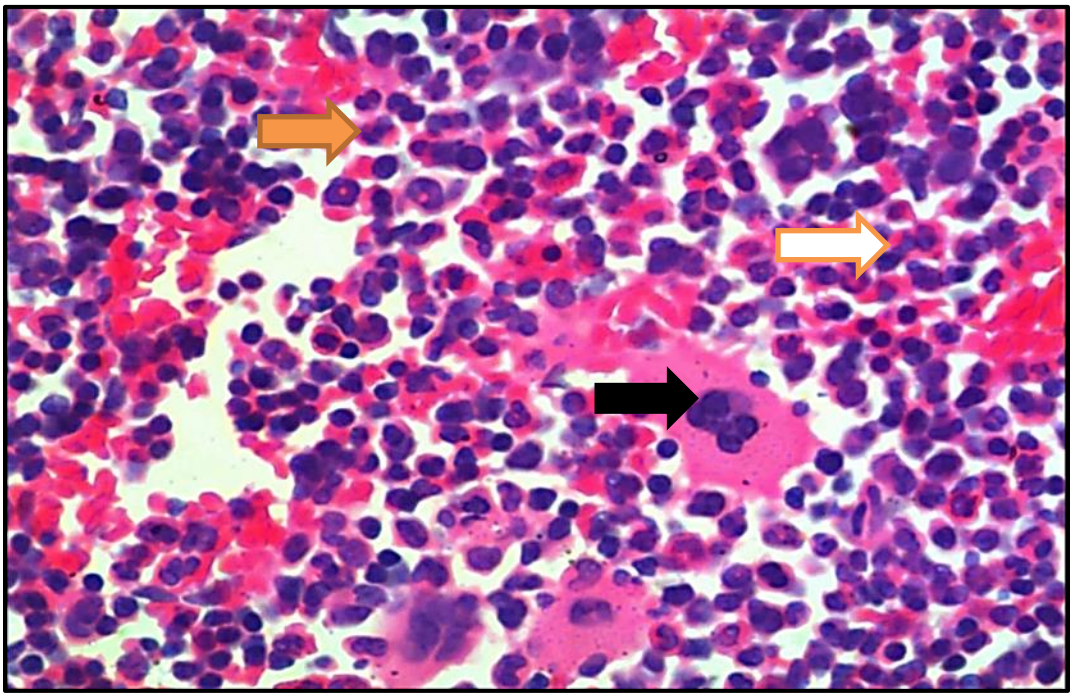

Figure 4-23: Histological section of bone marrow of rat femur bone marrow for treatment group (T5) shown normal architecture of bone morrow with improvement in cells proliferation (white arrow) mitotic activity (orange arrow) was also seen with presences of megakaryocytic cells (black arrow). H&E. stain , x400.

5. Discussion

5.1 GC-MS analysis for garlic extract

In our results of GC mass of garlic found the bioactive compound in garlic are mostly derived from sulfur-containing compounds and their precursor. These compounds include (precursors of Allicin because the allicin unstable degradation in high temperature, Diallyl sulfide, Allyl sulfide, 2- Vinyldithiins, and 3- Vinyl 1-1,2-dithiacyclohex. These compounds considers which are the main antioxidant biological activity for garlic (Shang *et al.,* 2019).

The antioxidant activity of these compounds also correlates with other bioactive compounds such as bioactive peptides, dietary fiber, polyphenols, and micronutrients (Capasso, 2013). This is in line with research conducted by Nencini *et al.* (2011), also our result agree with (Subroto *et al.,* 2021) in addition to containing bioactive organosulfur compounds, garlic also contains flavonoids and polyphenols, which are potential antioxidant agents.

The phenolic compounds from garlic have one or more hydroxyl groups that act as hydrogen donors (antioxidants) to neutralize free radicals. Antioxidants have been shown to safeguard the body from the effects of reactive oxygen species (ROS) and free radicals. ROS consists of several compounds, including hydrogen peroxide (H_2O_2), superoxide anions (O_2*****-), peroxyl (ROO-), hydroxyl (-OH), and alkoxyl (RO-) radicals are compound groups that will oxidize proteins, lipids, and DNA damage causing diseases (Abdel-Gawad *et al.*, 2014; Collin, 2019). Organosulfur and phenolic compounds as antioxidants in garlic play an important role in preventing cell and organ damage from the oxidation process (Capasso, 2013; Abdel-Gawad *et al.*, 2014).

The reaction of free radical scavenging by bioactive compounds in garlic can occur through several mechanisms. Garlic extract, especially from organosulfur compounds, stimulates the activity of glutathione peroxidase (GPX) and inhibits the decrease in the ratio of reduced to oxidized glutathione. GPX activity has also increased with the presence of diallyl disulfide (DADS) and diallyl sulfide (DAS). These compounds increase glutathione reductase activity and increase superoxide dismutase activity, while S-allylcysteine (SAC) and S-Allylmercaptosysteine (SAMC) increase reduced glutathione (GSH) synthesis. Old garlic extract, SAMC, and SAC showed radical scavenging activity, while DADS and DAS demonstrated selective action on diverse markers in testing their capability to react with free radical carbon tetrachloride. DADS also inhibits carbon tetrachloride-induced lipid peroxidation. Hence, the antioxidant properties of garlic may result from the contribution of various sulfur components at different process steps (Omar and Al-Wabel, 2010).

5.2 Characterization of Nanoparticles

5.2.1 Particle size (PS) analysis

The distribution, stability of solution, loading efficiency, range of drug release and cellular input in general are affected by the particle size (Souza *et al*., 2014). One of the most important factors influencing *in vivo* bio applications is the methods of cellular intake and internalization, especially endocytosis, which helps in the entry of particles and small molecules into the cell, as this method depends on the double layer of phospholipids of the cell membrane to help in active transport of materials assigned by engulf, and there are two methods of cellular intake according to the particle size of substances: phagocytosis, which ingests large particles with a diameter of 0.2-1μm, and pinocytosis, which involves the cellular intake of liquids, including dissolved particles of small diameter (Bertrand and Leroux, 2012).

The particle size test by laser in this study showed that the average particle size of garlic extract was 11.6 nm, while of CNP-G valued at an average size of 25.9 nm.

Bahari and Hamishehkar,(2016) pointed out that pharmakinetics, clearance, and distribution are greatly influenced by particle size in therapeutic delivery systems, where particle size greatly affects absorption, renal accumulation, and renal excretion, so it has been proven that particles that can enter or exit from the fenestrated capillaries both in the microenvironment of the tumors and in the endothelium of the liver and some tissues are (≤150nm). In addition, the nanoparticles of therapeutic delivery systems within the range (100-150 nm) remain in the blood stream and do not leave easily during perfusion and filtration of tissues, as occurs in the kidney, whose diameter of pores in its capillary lining is described as 60-100 nm, while the size of the pores in the

glomerulus is described as 10-15 nm, causing expulsion of very small diameter particles (20-30 nm).

As for very small sizes (less than 10 nm), they do not appear to have significance in the therapeutic delivery system because they are filtered into the capillary walls of the renal glomerulus and not reabsorbed again. According to the above, because nanoscale sizes of more than 150 nm may be exposed to phagocytosis by immune cells that remove them after accumulating in the liver and spleen, the presence of nanoparticles with sizes of 25-150 nm is able to stay for a longer period and the inability to escape through all types of capillaries, which ensure the safe availability and delivery of drugs (Kraft *et al*., 2014). So, the material in our study, showed appropriate particle size after loading on chitosan in the pharmakinetic and passive targeting in the Nano-therapeutic delivery system.

5.2.2 Zeta potential analysis

The surface charge of the nanoparticles has great importance in the therapeutic delivery system, as it affects the agglomeration of these particles and the adsorption state of the biomolecules and ions on their surface, which affects the ability of cells to absorb them, in addition to their biodistribution and the state of their exposure to attack by macrophages of the reticuloendothelial system, where the zeta voltage measurement is used to know the surface charge of the particles to indicate the state of stability in the solution (Honary and Zahir, 2013).

So, when the charge of the zeta potential is within the range ($^{+}$30 - $^{-}$30 mV), the solution with a pH becomes more likely to be problematic, the particles become less absorbed by the cells, and the toxicity of the solution may sometimes be generated within this range, which led the researchers to use other compounds such as binding substances to prevent particles from clumping together (Bi *et al*., 2017; Al-Saadi, 2020).

Therefore, the biologically preferred solution environment includes a zeta voltage higher than $^{+}$30mV or less than^{-} 30mV, which provides a high negative or positive charge for the particles and leads to an electrical repulsion of the particles from each other, which gives great stability to the suspension in the colloidal solution (Varsou *et al.,* 2020; Ramaye *et al*., 2021), as well as indicated that the charge^{-}30 mV is an ideal value in which the pH is neutral (7.2-7.4), the electrical repulsion is greater, which provides the spacing of particles from each other and avoids the occurrence of agglutination to suit greatly the internal environment of the body (Al-Dhabi and Arasu, 2018; Yedurkar *et al*., 2016).

The Zeta potential curves in the current study showed the surface charge of chitosan reached $^{+}$30.04mV and for TPP was $^{-}$19.62mV. The Zeta potential of garlic extract was $^{+}$28.08mV, while of CNP-G showed a charge elevated to $^{+}$48.43mV. The particles resulting from the binding of chitosan with TPP are characterized by the presence of a positive surface charge, which leads to binding strongly with all particles with a negative charge, which provides strong adhesion and direct attachment to the negatively charged surfaces (Vila *et al*., 2004). So, and some recent studies, Chitosan-TPP nanoparticles are characterized by high stability even within particles in different body fluid models (Gan *et al*., 2005).

According to the above, the Zeta potential of the therapeutic materials used in this study appeared at good levels within the normal range of the Zeta voltage, especially garlic extract, and it was observed that the use of hydrophilic polymers for nanoscale loading showed a significant improvement in the surface properties of the particles, including the surface charge (Honary and Zahir, 2013).

5.2.3 FTIR (Fourier transform infrared spectroscopy) analysis

Usually, the higher energy state of the infrared ray is excited after its absorption, where the absorption of certain frequencies of this radiation by molecules is quantitatively estimated at 20 kg/mol, which represents the possible change in it and which works on the stretch and bend of the covalent bonds (stretch and bend vibrational mods) of the molecule, which is called the vibrational patterns of the molecule, that are similar or matching to the normal state of the covalent bonds with the vibrations of the bonds of the infrared absorbing molecule only, where the amplitude of the vibrational motion is increased by the absorbed energy (Al-Saadi, 2020; AL-Muhna, 2017).

On the other hand, from the biological side, organic molecules contain functional groups that have the ability to absorb different infrared frequencies according to the type of group that distinguishes them from others. For example, the carbonyl group (C=O), which is characterized by the absorption of infrared radiation at the frequency of 1000 or 1700 cm-1, determines its exact value according to its location and its association with the atoms that are used to determine the composition of the organic molecule and its active groups (Al-Dulimi, 2020), where the peaks of the FTIR curve indicate that the vibration in IR absorption by the functional groups of the compounds relative to a certain wavelength led to a difference in the peaks of the FTIR curve, which showed different functional groups according to the type of material, characterized by the appearance of all or some the functional groups of the materials used in the final curve of the prepared solution confirms the success of their association within the same solution (Al-Kurdy, 2020).

According to the infrared spectrum of the therapeutic substances in our study, there are many peaks that may be attributed to the stretching or bending vibrations in the functional groups of these substances, but

observed in the spectrum of this substances slight change in the wavenumbers of some groups that may be attributed to the stretching and bending vibrations of the bonds, which shows a difference in the measurements of some peaks (Jabar, 2020; Al-Janabi and Al-Kalifawi, 2020).

Also, there are many peaks in the same nano-loading material (CNP-G), which confirms the presence of binding of materials in the nanoscale solution prepared in this study (Al-Saadi, 2020; Al-Dulimi, 2020). In addition, the presence of other groups may be attributed to the bonds generated from linkages of the functional groups of the prepared materials in nanoscale systems. So, FTIR spectra of the nano-prepared material (CNP-G), mention the correlation of the materials used in their preparation, where each material was confirmed by the presence of its functional groups, each according to its wavenumber in the solution, which indicates the presence of materials prepared for nano loading in the Nano therapy system that was used in this study.

5.2.4 X-ray diffraction (XRD)

The decreasing of crystallinity could be due to that CNP and CNP-G are composed of a dense network structure of interpenetrating counter ions of TPP, where the polymer chains crosslink with each other by TPP. Thus, the XRD pattern of CNP-G is characteristic of an amorphous polymer. The mean crystalline size of the synthesized CNP-G is 25.9 nm based on the most intense diffraction peak FWHM at 36.28°. Earlier studies also acquired similar X-ray diffraction patterns for green synthesis of zinc oxide nanoparticles (Vaishnav *et al*., 2017) .

Similar findings have also been reported (Talam *et al.,* 2012). These diffraction peaks have also been detected to expand attributable to small particle sizes in nano-scale with a moderate crystallinity of 36.29

percent. The dry powders of the CNP-G was used for X-RD analysis. The diffracted intensity was documented at 2theta angles from 27 ° to 37 °.

5.3.5 TEM (Transmission electron microscope) analysis

The transmission electron microscope analysis was used to determine the shape and size properties of nanoparticles, including the particle shape and nanoscale, in addition to the isolation or accumulation of particles with each other and the general appearance of their connection after the preparation and adsorption of therapeutic materials. Many forms of nano-loaded material appeared in TEM image in this study, which can be attributed to the idea that the polymer layers and therapeutic material were difficult to see under the electron microscope due to their low electronic density (Bachelet, 2017).

The particles have different shapes, such as spherical, round, oval, and irregular, but most of them are spherical in shape. It is clear that the material, which contains complex functional groups (amine oxide, hydroxyl, and carboxylate), plays a major role in the formation of various shapes of nanoparticles under the conditions of the present experiment (Premkumar *et al*., 2011). Hence, the specific interaction between the functional groups of chitosan and the substances at different surface levels resulted in different forms of conjugation.

5.3 *In vivo* experiment

5.3.1 Hematological parameters

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

Results of the current study showed a direct effect of Phenylhydrazine (PHZ) in creating anemia, wherein a decrease in hemoglobin concentration, PCV , and red blood cells count in T1. It has been demonstrated in several studies, that there were a significant association of diagnostic values between RCB, Hb, PCV and blood indices in rats when exposed to PHZ.

In conducted study 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 with a significant increase 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 treated with 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 other study (Beshel *et al*., 2018), the results of the study 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 ; Berger, 2007).

As such, these free radicals lead to reactive oxygen species flow into the blood system, causing damage in membranes and decomposing red blood cells through oxidation of unsaturated fats in membranes and generation lipid peroxidation, thus the membrane loses its role of maintaining cells' functions and their lives continuity (Ogbe *et al*., 2012; Ali *et al*., 2014).

Shukla and Singh (2015) were indicated that the accumulation of free radicals in blood cells leads to damage to the nuclei inside them, causing anemia , a decrease in the RBC count, an increase in WBC, and a decrease in the size of red blood cells along with the level of cells' hemoglobin, this is due to the PHZ high toxicity which has a direct effect on cells (Ashour, 2014), it was also observed in current study.

Giving doses of garlic extract and CNP-G to rats, as shown in table 4-2 , 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 an inhibiting 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 (Ranjan and Vats, 2016; 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 CNP-G 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

erythropoetin (a potent stimulator of the bone marrow) (Shalaby *et al*., 2006; Suha , 2014).

Moreover, it has been found that garlic has some constituents(such as organic sulfur and phenolic) that may play a role in the function of organs related to blood cell formation such as thymus, spleen, and bone marrow (Fazlolahzadeh *et al*., 2011).

5.3.1.2 Red blood corpuscles indices

In the present study the induction with phenylhydrazine (PHZ) caused increased values of MCV, MCH, and MCHC, as observed in table 4-3. Erythrocytes with normal of haemoglobin concentration (MCHC) are normochromic whereas, abnormally high and low mean values indicate hyperchromic and hypochromic conditions respectively, though there is no hyperchromic condition (Igwe *et al*., 2020) these results are partially agreement with our current study. So the MCV, MCH and MCHC values in this work were suggesting macrocytic hypochromic anemic condition.

These result contrasted with those of Tchogou *et al*., (2016) which obtained RBC indices decrease after phenylhydrazine injection but they were in agreement with Ndem *et al*., (2013) and Ponmozhi and Ramya(2015). The effect of garlic extract and CNP-G on RBC indices showed a significant decrease especially in T4 and T5 . So, it is assumed that the increase of blood indices may be attributed to a defense reaction against *Allium sativum*, which occurs by stimulation of erythropoiesis (Fazlolahzadeh *et al*., 2011).

5.3.1.3 Platelets count and ESR

Results of the current study showed a direct effect of anemia on platelets count in T1 compared with negative control and all treatment groups. Scharbert *et al*. (2011) that reported a significantly increased in velocity of platelets aggregation in anemic blood sample as result of low hemoglobin concentration.

The increase in platelet count observed in T1 is also in line with the findings of the studies of Akinbami *et al*.(2012), Chinawa *et al*.(2013) were they collectively revealed an increase in platelet count in anemic patients compared with control. The increase in platelet count may be as a result of functional asplenia with a decreased in platelet pooling in the splenic population as postulated by Ataga and Orringer (2003), and may be increase platelets due to increase of megakaryocyte in bone marrow.

The platelet count showed a significant decrease in all treatment groups that received extract and CNP-G. Plants of the genus *Allium* such as onion and garlic are often consumed as a source of compounds(organic sulfur) which inhibit human platelet activity. Antiplatelet activity of these plants is in part due to the concentrations of organosulfur compounds (Lorigooini *et al*., 2014). The aqueous extracts of garlic inhibit platelet aggregation induced by collagen and arachidonic acid in a dose-dependent manner *in vitro*, furthermore, raw garlic was found to inhibit cyclooxygenase activity in a non-competitive, dose-dependent and irreversible manner (Rahman, 2007).

Also, the ESR recorded a significant increase in anemic group as compared with other groups in present study that agreement with Ibrahim *et al*.(2014) who recorded anemia increase ESR there was negative correlation between ESR and PCV. In anemia , with the haematocrit

reduced, the velocity of the upward flow of plasma is altered so that red blood cells sediment faster (Alao, 2010). Other factors may also affect ESR red cell changes that are especially prone to effect ESR. If hematocrit is reduced, red cell aggregates sediment faster. The more severe the anemia the higher the ESR (Hale *et al*., 2019).

After period of treatment (60 days) of rats in anemic group observed decrease in level of ESR. Garlic can be considered as a useful natural herb in inhibition of inflammation. The advantageous effects of garlic on health are due to organosulfur compounds in it (Schafer and Kaschula, 2014). Ban *et al*.(2009) revealed that thiacremonone, a sulfur compound from garlic, prohibits Nuclear Factor Kappa B NF-kB activation through interacting with sulfhydryl group of nuclear factor kappa B molecules. The result of Asgharpour *et al*.(2021) also demonstrate a significant decrease in inflammatory biomarkers such as ESR by using the garlic extract which is agreement with the results of the present study.

5.3.1.4 The leukocytes count

The results of the current study, as shown in table 4-5, show significant differences in the numbers of WBCs was observed a significant increase in the numbers of white blood cells of anemic group compared to the control group . In addition, in the case of infections, the bone marrow releases a type of cells called immature cells since the bone gets the stress of producing new cells .This causes an increase in the white blood cells total number (Wershana, 2000).

Regarding to deferential WBC changes, PHZ injected rats showed increased in monocytosis, granulocyte, and lymphocyte

comparing to control rats. These results agree with Bansode *et al*.(2019). White blood cells are defensive mechanisms used by the body to combat cell infiltration by foreign agents or infections; thus the increased proliferation may indicate an immunological response due to acute infections, cell damage or inflammation (Atawodi *et al*., 2011) which might have been caused by the introduction of toxic substance such as PHZ (Baker *et al*., 2021).

The increased in lymphocyte at garlic extract and CNP-G (T3 and T5) groups compared with control group came into agreement with the earlier reports in that supplementation of garlic had significantly improved leukocyte count, indicating the immunostimulant properties of garlic (Fazlolahzadeh *et al*., 2011). Immunostimulants attached to specific receptors on the cell surface of the phagocytes and lymphocytes activating this cell to produce some enzymes that can destroy pathogens . Many defense mechanisms activated by garlic counteract the challenge infection including the production of superoxide anions against infections (Suha , 2014).

Moreover, the increased in lymphocytes and neutrophils counts at garlic extract and CNP-G groups may be related to an increase in the production of some chemical cytokines (interferon, interleukins and complement proteins). These cytokines stimulate other arms of the immune system and increase the activity of natural killer cells as well as T- and B- lymphocytes (Oluwole , 2001 ; Iranloye, 2002; Thanikachalam *et al*., 2010). Furthermore, it has been found that garlic contains a therapeutic factor (Germanium) which enhances natural killer cell and macrophage activity in experimental animals that stimulate the immune function (Fazlolahzadeh *et al*., 2011; Suha ,2014).

5.3.2 Measuring of Biomarkers parameters

5.3.2.1 Total serum proteins, albumin, and fibrinogen

Intrapertoneal injection of PHZ generated a significant 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 total protein and albumin 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. Study have shown that intravascular hemolysis in any condition may damage the liver and other vascular organs (Onyeaba *et al*., 2017) apart from hemolysis induced liver injury.

After treatment with garlic extract and CNP-G observed increase in total protein and albumin .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%. Also after treatment with garlic extract and CNP-G fibrinogen level decrease, our results are in 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 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).

5.3.2.2 Electrolytes of blood (Na^+, K^+, Ca^{++}, Iron)

In this study, observed that serum sodium levels were significantly lower and serum potassium levels were significantly higher in anemic group when compared with negative control group. This result agreement with Rajagopal *et al*., (2018); Shraf *et al*., (2017); and Agoreyo and Nwanzen (2010). Previous study 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 *et al*., 2014).

study have shown variation in the erythrocyte membrane Na^+-K^+ ATPase activity was modulated by the changes in the differences resulting from hematological disorders (Omar *et al*., 2017). Furthermore, an increase in red cell membrane permeability to sodium or potassium has been described in a variety of red cell disorders. Previous study have stated that Na^+-K^+ ATPase activity is higher in the primary anemia patients. This elevation may compensate the mechanism for adaptation of the patients with low oxygen and its physiological role in the cell (Rajagopal *et al*., 2018).

The results obtained after treatment with garlic extract and CNP-G showed increase in both Na^+ and K^+ especially in (T5) when compared with anemic group this may be because the highly content of garlic from potassium and the converted garlic to polymer NPs increase the bioavailability and bioactivity (Lu *et al*., 2021). 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 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 *et al*., 2010) that agreement with present study.

After treatment with garlic extract and CNP-G the concentration of calcium increase in all treatment groups and highly increase in T2 and T5 as compared with anemic group and negative control group this high increase in T5 may be due to the garlic contain calcium and convert garlic to NPs improve the bioavailability of garlic (De Greef *et al*., 2020).

This result agrees 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. This results could be explained by a

total iron passage across the enterocytes apical membrane transporters divalent metal transporter 1(DMT1) to the blood (Bleackley *et al*., 2009).

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 CNP-G 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) suggesting 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.

5.3.3 Measuring of antioxidant and oxidation status

4.3.3.1 Total antioxidant capacity (T-AOC) and malondialdehyde (MDA)

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 Bansode *et al*., (2019). Also, Ashour, (2014) reported that PHZ induced significant decrease in GSH level. As PHZ induced increase in reactive oxygen species (ROS) and lipid peroxidation with decrease glutathione.

current results also in agreement with Kolawole *et al*.,(2017) who found 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.

According to Ryan and Prescott (2010), 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 (2010), 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 (Perveen *et al*.,2015). 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 (Capasso, 2013).

Comparing to anemic group, treatment groups with garlic extract and CNP-G showed increase in total antioxidant capacity and decrease in malondialdehyde levels. These results agree with Al-sayed *et al*.,(2017) and Al-Fayyadh and Wadood(2021), who found 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 (Zaidi *et al*., 2015).

Also, these result agree with Ghorbel *et al*.,(2015) 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*.,(2009), 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 radicall, thus enhance in vivo endogenous antioxidant system and prevent oxidative stress (Chung *et al*., 2006).

5.3.4 Biomarkers to evaluation blood (HPX, EPO, VB12, and COX-1)

5.3.4.1 Hemopexin (HPX)

In present study the HPX recorded significant reduction in anemic group as compared with control and treatment groups. The diminished of hemopexin level in hemolytic anemia group is compatible with previous studies that indicate levels of hemopexin are reduced as those of heme rise, thus concentration of this protein is, indicating that good monitor for assessment of severity of intravascular haemolysis and activity in heme scavenger (Al-Ghurabi and Al-Rawaziq, 2016) .

Also previous studies refers to Hpx is regarded as second line of defense against intravascular hemolysis associated with hem in release from Hb-$Fe3^{+}$ (Tolosano *et al*., 2010 ; Hanson *et al*., 2011). Hemopexin scavenging of free heme from the plasma and limits the amounts thus preventing oxidative damage by radical formation in the circulation and contributing to the conservation of body iron. The presence of heme in plasma which is produced from the oxidation of hemoglobin released through the destruction of erythroblasts in conditions intravascular hemolysis (Ascenzi *et al*., 2005; Al-Ghurabi and Al-Rawaziq, 2016).The HPX deficiency results in increased oxidative stress and proinflammatory, under oxidative stress, hemoglobin can release the hem. HPX and Hp are acute phase proteins produced by the liver with the highest binding affinity for hem (Mehta *et al*., 2016).

When treatment with garlic extract and CNP-G recorded significant elevated in HPX level in treatment groups. This may be due to the decrease level of hem that result of treatment hemolytic anemia. Also the HPX level increased in treatment groups may be due to the garlic reduced the oxidative stress level (Al-sayed *et al*., 2017).

To limit free heme availability and prevent free radical formation, mammals use HPX as the major heme scavenger protein. HPX binds to free heme, and the resultant heme-HPX complex is taken up by macrophages and hepatocytes through the CD91 receptor via receptor-mediated endocytosis, thus the HPX level increased to elimination of heme (Delanghe and Langlois, 2011).

5.3.4.2 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 (Pagani *et al*., 2019).This leads to suppression of hepcidin transcription by erythroferrone(ERFE), an EPO target gene produced by erythroblasts (Kautz *et al*.,2014), by molecules (e.g., PDGF-BB) released by other tissues(Sonnweber *et al*.,2014).

Current study agree with Jiang *et al*.,(2016) who found that serum EPO content was increase almost 4000 fold at the first 12 h, compared to untreated control. Under conditions of stress erythropoiesis, elevated EPO levels are associated to suppression of hepcidine synthesis, a response that increase iron absorbtion and iron egress to provide sufficient iron for production and maturation of erythroid cells (Gammella *et al*., 2015; Rainville *et al*.,2015). Also, PHZ may effect on EPO receptors of JAK-

STAT pathway which is responsible for the maturation of red blood cells. After PHZ- induced anemia, EPOR-HM mice failed to respond with efficient splenic stress erythropoiesis. Defects in the erythropoietin receptor may produce erythroleukemia and familial erythrocytosis(Singh *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.

5.3.4.3 Vitamin B12 (VB12)

The present study showed significant decrease of VB12 in anemic group compare with other groups. Human B12 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 (USDA, 2018 ; Qiu *et al.*, 2019).

5.3.4.4 Cyclooxygenase (COX-1)

Cyclooxygenase plays a key regulatory role in prostaglandin synthesis and occurs in both forms, COX-1 is expressed in a wide variety of tissues, while COX-2 has a more limited distribution. In the present study, it is found that COX-1 highly significant increase in anemic group as compared with negative control and treatment groups. These may be because the COX induction in bone marrow and spleen may play a role in hematopoiesis that is associated with antineoplastic therapies (Lorenz, *et al*., 1999). COX-1 may be have positive role in repair damage in bone marrow may be act as antineoplastic.

Garlic inhibiting cyclooxygenase activity and thus thromboxane A2 formation, by suppressing mobilization of intraplatelet Ca^{+2}, and by increasing levels of cAMP and cGMP (Rahman, 2007). These results agree with our results that recorded decrease in COX level in treatment groups when administration extract and NPs of garlic. Also, the 2-propenyl thiosulfate from garlic affected on cyclooxygenase activity in a dose-dependent manner (Chang *et al*., 2005).

5.3.5 Biomarker to evaluation toxicity (serum amyloid SAA)

Serum amyloid A is a family of acute-phase proteins named after a disease. It is known as a biomarker of inflammation (Zhang *et al*., 2019). In our study, we estimate the serum amyloid level to investigation of toxicity of nanoparticles and effects of nanoparticles in the body. In our results found elevated of SAA in anemic group when compared with all other groups, but recorded significant decrease in treatment groups. This may be because not to use metals in preparation of garlic nanoparticles,

as chitosan was used in preparation of CNP-G. Chitosan-based nanomaterials are on the forefront and attract wide interest due to their versatile physicochemical characteristics such as biodegradability, biocompatibility, and non-toxicity, which play a promising role in biological applications (Rizeq *et al*., 2019).

Induction of SAA1 in response to air pollution and nanoparticles has also been reported earlier in other studies (Halappanavar *et al*., 2011). Hadrup *et al*.(2019) found that pulmonary exposure to ZnO nanoparticles by inhalation or by instillation induces a pulmonary acute phase response. There are a limited number of occupational biomonitorig studies which included assessment of exposures to nanoparticles and measurements of serum amyloid A or C-reactive protein (Hadrup *et al.*,2020). Inhalation of occupationally relevant doses of ZnO increased SAA level by a factor of 10 (Monse *et al*., 2018).

Also, Ng *et al*.,(2018) observation that AuNPs have the propensity to stimulate SAA1 production in lung epithelial cells, suggests that AuNPs induced an inflammatory response. Moore *et al*., (2017) have studied the effect of AuNPs properties on inhibition of beta amyloid aggregation, but when preparation biopolymer-coated AuNPs by using chitosan recorded low toxicity, higher dispersibility, availability of function groups, and robust chemical and thermal stability, also investigated the toxicity of biopolymer-coated AuNPs-insulin amyloid fibers on pancreatic(PaTu-T and PaTu-S) and cell lines compared to pure insulin amyloid fibrils, all other types of biopolymer-coated AuNPs and AuNPs-insulin amyloid fibrils present a lower cytotoxicity (Meesaragandla *et al*., 2020).

5.3.6 Histological study

5.3.6.1 Histological evaluation of femur bone marrow

Histopathological evaluation indicated that anemia-induction by phenylhydrazine administration caused pathological changes of moderate grade in bone marrow tissues the present results agree with (Shetha *et al.*, 2021). The presence of huge space in bone marrow of anemic group may be because of destroyed of cells that produce erythrocyte and replased by adipose tissue resulting hypoplasia of bone marrow.

These pathological changes were reversed by garlic extract and CNP-G 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 CNP-G 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,(2008) 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 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 (Ogbe *et al.,* 2010). The GC mass analyses of garlic showed the presence of highly sulfur compounds or other active compounds that may be responsible rot the observed antianemic activity of garlic.

Conclusions

1. The garlic extract possesses a therapeutic efficacy as an anti-hemolytic anemia in rats exposed to PHZ via detoxification of active metabolites and their antioxidant activity that give good chance for tissue repair. There was a clear protective role of garlic extract against the anemia.
2. Garlic extract loaded chitosan nanoparticles more efficient than garlic alone to improvement the blood parameter of anemic animals induced with PHZ.
3. The presence of new substances within the garlic extract during the examination of the GC-MS such as (carbamaldehyde, 1-propen, 1-bromo, 2-ethylpyrrole, methacrylic anhydride, and 2-propynenitrile,3).

Recommendations:

1. Using chitosan nanoparticles to load other substances.
2. Quantitative and qualitative estimation of secondary metabolism compounds for the purpose of showing the possibility of adopting them as a source of natural antioxidants and thus identifying the possibility of investing in the garlic plant and expanding its cultivation.
3. It was recommend through this study, more research, including molecular studies, is required about the protective role of garlic against other diseases.
4. Study of the side effects of loading plant extract on nanoparticles.

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