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Theme

Preparation And Characterization Of Immobilized Camel Milk Proteins On Zinc Nanocomposites And The Study Of Their Effect Against Experimental Anemia In Rats

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بسم الله الرحمن الرحيم

إهـــــــــداء

خـرّيجة أمضي ودربي ساطع تشدو به الآمال في وجداني

خـرّيجة أمرنو إلى درب العلا بعزائم الأقدام والإيمان

إنّي قطعت العهد أن أرقى السما أزهو وروح الجد في شرياني

خريجة انا ولولا توفيقي من عند الله ثم دعم عباده لما تخرجت

الى صاحب السيرة العطرة و الفكر المستنير الى "**والدي**" الحبيب

الى قرة عيني و من افضلها على نفسي الى "**امي**" الحنون

الى "**دعاء**" و "**لجين**" العضد و السند و مصدر الحب و الدعم

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لكل فرد من **عائلتي** صغيرا كان او كبير

لأصدقائي كل بمكانه في قلبي

لرفيقة الدرب "**آية**" بهية الخلق و الاخلاق

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يرزقكم من خيراته الرزق الوفير في الدنيا و الفردوس الأعلى في الاخرة .

- وجـــدان -

إهـــــــــداء

أنا لست ممن قال كان أبي . . بل قلت للأكوان ها انا ذا"

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Abstract

This work investigates the preparation of immobilized Camel milk (CM) proteins on zinc nanocomposites (ZnNPs) and the study of their effectiveness against physiological, biochemical, and histological disturbance induced by experimental Anemia in rats. *In-vitro* study was conducted on immobilized proteins on zinc oxide nanoparticles to characterize and analyze their anti-inflammatory and antioxidant properties using standard analytical methods and protocols. *In-vivo* study, twenty male albino Wistar rats were randomly divided into 4 groups (n=5); healthy rats (Control group), PHZ Anemia rats (PHZ group), PHZ Anemia rats treated with casein milk protein zinc nanoparticles (CP-ZnNPs group), PHZ Anemia rats treated with whey milk protein zinc nanoparticles (WP-ZnNPs group). Inducing anemia in rats was achieved using the chemical compound phenylhydrazine (40mg/Kg) via intraperitoneal injections. Both types of treatments were administered to the rats using the same route for three weeks (21 days). Various *in-vivo* study parameters were assessed, including hematological, biochemical, oxidative stress markers and the histopathology study of the spleen and kidney tissues.

Characterization results of ZnNPs confirmed a fort immobilization of CM proteins (Casein and whey) with an oval and multifaceted shape, a size relatively between 220 and 390 nm, also an immobilization yield 80.68% for CP-ZnNPs and 63.71% for WP-ZnNPs. On the other hand, results of biological activities revealed that CP-ZnNPs had a higher antioxidant activity than WP-ZnNPs; likewise, WP-ZnNPs illustrated a higher anti-inflammatory activity than CP-ZnNPs.

Moreover, *in-vivo* part's results have demonstrated a significant change in comparison of PHZ with treated groups concerning hematological and biochemical parameters along with a significant shift detected in oxidative stress markers which indicates the healing impact of CP-ZnNPs and WP-ZnNPs. Finally, histopathological examination illustrated the complete recovery for the CP-ZnNPs group shown in kidney and spleen sections of rats treated with a partial improvement detected in WP-ZnNPs treated rats.

Eventually, the current study confirmed that the combination of Camel milk proteins, precisely casein, and whey with zinc oxide nanocomposites has a potent effectiveness in treating anemia, as well as reducing symptoms due to their antioxidant and anti-inflammatory properties.

Key words : Anemia, Phenylhydrazine, Camel milk, Oxidative stress, ZnNPs, Rats.

الملخص

الهدف من هذا العمل هو تحضير بروتينات مستخلصة من حليب الإبل مثبتة على مركبات الزنك النانوية (ZnNPs) ودراسة فعاليتها ضد الاضطرابات الفسيولوجية والكيميائية الحيوية والنسيجية الناتجة عن فقر الدم التجريبي في الفئران. باستخدام الطرق والبروتوكولات التحليلية القياسية، تم إجراء دراسة مختبرية على جسيمات أكسيد الزنك النانوية لتحليل خصائصها الفيزيائية والكيميائية وايضا دراسة تاثيراتها المضادة للالتهابات والمضادة للأكسدة، وتحديد كمية البروتينات المثبتة فيها.

من جهة اخرى قمنا بالدراسة على الحيوانات حيث تم استخدام عشرين فأراً من ذكور الوستار البيضاء قسمت بشكل عشوائي إلى 4 مجموعات(n=5); فئران سليمة (مجموعة شاهدة)، فئران مصابة بفقر الدم (مجموعة PHZ)، فئران مصابة بفقر الدم ومعالجة بجسيمات الزنك النانوية المحتوية على بروتين الكازين (مجموعة CP-ZnNPs)، وفئران مصابة بفقر الدم ومعالجة بجسيمات الزنك النانوية المحتوية على بروتين مصل الحليب (مجموعة WP-ZnNPs)، تم تحفيز فقر الدم في الفئران باستخدام مركب فينيل هيدرازين الكيميائي (40 ملغ/كغ) عن طريق الحقن داخل الصفاق. تم إعطاء كلا العلاجين للفئران بنفس الطريقة لمدة ثلاثة أسابيع (21 يومًا) حيث تم تقييم العديد من المعايير البيولوجية في أجسام الفئران، بما في ذلك المعايير البيوكيميائية ومكونات الدم، معايير الإجهاد التأكسدي، ودراسة علم الأمراض النسيجي لأنسجة الطحال والكلى.

كشفت نتائج دراسة الخصائص الفيزيائية والكيميائية لجسيمات الزنك النانوية (ZnNPs) تثبيتًا قويًا لبروتينات حليب الإبل (الكازين والمصل) مع تشكل الجسيمات بشكل بيضاوي ومتعدد الأوجه، وحجم يتراوح بين 220 و390 نانومتر؛ بالإضافة إلى ذلك، أظهرت جسيمات الزنك النانوية المحتوية على بروتين مصل الحليب (WP-ZnNPs) نشاطًا مضادًا للالتهابات أعلى من جسيمات الزنك النانوية المحتوية على بروتين الكازين (CP-ZnNPs) وكذلك أن جسيمات الزنك النانوية المحتوية على بروتين الكازين (CP-ZnNPs) أظهرت نشاطًا مضادًا للأكسدة ومستوى بروتين أعلى من جسيمات الزنك النانوية المحتوية على بروتين مصل الحليب (WP-ZnNPs) .

. أظهرت نتائج هذا الجزء تغييرًا كبيرًا عند مقارنة مجموعة PHZ بالمجموعات المعالجة فيما يتعلق بالمعايير البيوكيميائية ومكونات الدم، بالإضافة إلى تحول كبير تم اكتشافه في معايير الإجهاد التأكسدي، مما يشير إلى التأثير العلاجي لجسيمات الزنك النانوية المحتوية على بروتين الكازين (CP-ZnNPs) وجسيمات الزنك النانوية المحتوية على بروتين مصل الحليب (WP-ZnNPs). أخيرًا، أوضح الفحص النسيجي وجود تحسن كبيرلمجموعة الفئران المعالجة بجسيمات الزنك النانوية المحتوية على بروتين الكازين (CP-ZnNPs) في مقاطع الكلى والطحال، مع تحسن جزئي تم اكتشافه في الفئران المعالجة بجسيمات الزنك النانوية المحتوية على بروتين مصل الحليب(WP-ZnNPs).

في النهاية، أكدت الدراسة الحالية أن الجمع بين بروتينات حليب الإبل، تحديدًا الكازين والمصل، مع مركبات أكسيد الزنك النانوية له فعالية قوية في علاج بعض التغيرات الفيزيزلوجية و البيوكيميائية الناجمة عن فقر الدم وتقليل الأعراض بفضل خصائصها المضادة للأكسدة والمضادة للالتهابات.

الكلمات المفتاحية: فقر الدم، فينيل هيدرازين (PHZ)، حليب الإبل، الإجهاد التأكسدي، جسيمات الزنك النانوية، الفئران.

ABBREVIATIONS LIST

(ACE) : Angiotensin-converting enzyme

(AI) : Anemia of inflammation

(ALAT) : Alanine aminotransferase

(ASAT) : Aspartate aminotransferase

(ATP) : Adenosine triphosphate

(BS) : Bawman's space

(C) : Carbon

(CA) : central arteriole

(CAT) : Catalase

(CM) : Camel milk

(CPP) : Caseinophosphopeptides

(CP-ZnNPs) : Casein protein zinc nanoparticles

(Cu) : Copper

(•CH2CH3) : Ethyl

(•CH3) : Methyl

(DNA) : Deoxyribonucleic acid

(DPPH) : 1,1-diphenyl-2-picrylhydrazyl

(DT) : Distal tubules

(DTNB) : dithio-bis-2-nitrobenzoic acid

(EDTA) : Ethylenediaminetetraacetic acid

(EDX) : Energy dispersive X-ray

(EPO) : Erythropoietin

(ERFE) : Erythroferrone

(FNS) : Numération formule sanguine

(FTIR) : Fourier transform infrared spectroscopy

(G) : Glomerulus

(GI) : Gastrointestinal

(Gly) : Glycemia

(GPx) : Glutathione peroxidase

(GRx) : Glutathione reductase

(H) : Hemorrhage

(H_2O_2) : Hydrogen peroxide

(Hb / HGB) : Hemoglobin

(HCT) : Hematocrit

(HO1) : Haemoxygenase

(•H) : Hydrogen atom

(I) : Inflammation

(IC50) : Half maximal inhibitory concentration

(ID) : Iron deficiency

(IDA) : Iron deficiency anemia

(IY) : Immobilization yield

(LABs) : Lactic acid bacteria

(LDH) : Lactate dehydrogenase

(MCH) : Mean corpuscular hemoglobin

(MCHC) : Mean corpuscular hemoglobin concentration

(MCV) : Mean Corpuscular Volume

(MDA) : Malondialdehyde

(Mn) : Manganese

(MZ) : Marginal zone

(N) : Necrosis

(N) : Nitrogen

(NPs) : Nanoparticles

(NS) : No significance

(•NO) : Nitric oxide

(O) : Oxygen

(O2 •−) : Superoxide

(•OH) : Hydroxyl

(OD) : Optical Density

(p) : p value

(P) : Phosphorus

(PHZ) : Phenylhydrazine

(PLT) : Platelets

(PT) : Proximal tubules

(RBCs) : Red blood cells

(rmIL-3) : Recombinant mouse interleukin 3

(RNA) : Ribonucleic Acid

(RNOS) : Reactive nitrogen oxide species

(RNS) : Reactive nitrogen species

(ROO•) : Peroxyl

(ROS) : Reactive oxygen species

(RP) : Red pulp

(S) : Sulfur

(Se) : Selenium

(SEM) : Scanning electron microscope

(SEM) : Standard error mean

(SOD) : Superoxide dismutase

(TBS) : Tris-buffered saline

(TC) : Total cholesterol

(TFR1) : transferrin receptor

(TG) : Triglyceride

(TLRs) : toll-like receptors

(TNB) : 2-nitro-5-mercocapturic acid

(TNF-α) : Tumor Necrosis Factor Alpha

(UV-VIS) : Ultraviolet-visible spectroscopy

(WBCs) : White blood cells

(WHO) : World Health Organization

(WP- ZnNPs) : Whey protein zinc nanoparticles

(WP) : White pulp

(Zn) : Zinc

(ZnONPs) : Zinc Oxide Nanoparticles

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SUMMARY

Introduction

Introduction

A range of health problems can arise from anemia disease. Anemia is considered the most widespread disorder worldwide. This global public health issue which affects both developed and developing countries with major consequences for human health and their social and economic development (WHO 2005).

In a healthy subject, a state of nutritional balance exists when the amount of food eaten is equal to the amount of nutrients for the proper functioning of the body on one hand .On the other hand, This equation can be imbalanced when there is a decrease in nutrition, absorption and utilization along with an increase in losses and needs. It is a result of deficiency of one or more essential nutrients in the body, especially iron as known that it is one of elements that contributes in the formation and development of red corpuscles and the synthesis of hemoglobin along with other minerals, i.e., zinc, copper, magnesium, cobalt, molybdenum, vitamins, especially folic acid and vitamin B12,and amino acids. When these are exhausted, all the bodily functions in which this nutrient plays a part are affected. Anemia is caused by the lack of red blood cells or hemoglobin. The body unable to produce enough healthy red blood cells. Hemoglobin is the protein molecule in RBCs that transport oxygen to the body tissues. Since hemoglobin carries oxygen from longs to capillaries , anemia leads to hypoxia. Human beings need oxygen for their survival , anemia can have wide range of health problems .So, deficiency of RBC reduces the amount of oxygen available to the body (Bhadra & Deb, 2020).

According to the WHO, anemia is defined as having hemoglobin levels lower than 12.0 and 13.0 g/dL in females and males, respectively (Safiri *et al.,* 2021). Anemia remain undetermined in people & symptoms can be minor. The signs and symptoms can be related to the underlying cause or the anemia itself. Most commonly, people with anemia report feelings of weakness, or fatigue, and shortness of breath. In very severe cases of anemia, the body may compensate for the lack of oxygen-carrying capability of the blood by increasing cardiac output. Anemia is more prone in women of reproductive age (14 - 45 years old) and children. Women require special nutrition because of their period of menstruation and pregnancy which can lead to a heavy blood loss in addition to lactation. (Gotapagar *et al.,*2016).

As known that Camel milk has various health benefits (Zahra *et al.,* 2021) and a high potential to promote functional health (Amrouche *et al.,* 2020)due to its richness in proteins , lactose , vitamins and a variety of minerals (Fufa & Haile, 2020). that could lead to its

capacity in healing different health problems such as Diarrhea , Autism and Lactose Intolerance in children (Akbar *et al.,* 2020) and its diabetes control therapy along with its effects as Anti-hyperlipidemic Agent (Fufa & Haile, 2020).

zinc oxide, inorganic chemical, for its protective vital role in preventing pathological changes in cells (Liu *et al.,* 2023) , as well as modifying their structure and size resulting in a unique and improved properties, such as particle size distribution and morphology, that are not seen in larger particles of bulk material (Jamkhande *et al.,* 2019). This process is utilizing a green synthesis way that involves constructing nanomaterials in a clean, safe, cost-effective, and environmentally friendly manner that often consists of specific enzymes, amino acid groups, proteins, or chemical structures which can be aimed for minimizing risks to human health and the environment in comparison to the traditional ways (Dkhil *et al.,* 2020).

In order to be a part in treating anemia , our study focused on using natural based product in this case freshly brought Camel milk proteins , precisely talking Casein and whey proteins . For the purpose of better healing the targeted health issue , we have combined these proteins with zinc oxide nanoparticle as way of proteins immobilization by using nanotechnology techniques to finally form Casein protein-zinc nanoparticles (CP-ZnNPs) and whey protein-zinc nanoparticles (WP-ZnNPs).

Taking into consideration all the previous outcomes, we have found that the most suitable way to maintain a comprehensive study is through conducting the two following methods :

***In-vitro* part :** it consists of extraction the Camel milk proteins (Casein and whey), preparation of zinc nanoparticles along with quantitative and qualitative characterization of these compounds and evaluation of their biological property.

***In-vivo* part :** it was about evaluation of the therapeutic efficiency of CP-ZnNPs and WP-ZnNPs against metabolic, physiological and histological changes induced by experimental Anemia using PHZ in rats.

First part:

Bibliographic synthesis

Chapter I

Anemia & Phenylhydrazine

1. Defining anemia

Anemia is 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, affects roughly one-third of the world's population. It 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 & Suchdev, 2019). Hemoglobin thresholds to define anemia were first proposed by the World Health Organization (WHO) in 1959 (Garcia-Casal *et al.*, 2019).

According to WHO criteria, anemia was defined as hemoglobin <12.0 g/dL in women and <13.0 g/dL in men. However, it differs by age, sex, and pregnancy status as shown in (Table 1) (Gabriele Masini *et al*., 2022).

Table 1: World Health Organization criteria for anemia (Saxena *et al*, 2018).

	Venous blood (gm/dL)	**MCHC**
Adult males	13	34
Adult females, nonpregnant	12	34
Adult females, pregnant	11	34
Children (6 months–6 years)	11	34
Children (6–14 years)	12	34

Anemia can result from multiple causes, including nutritional deficiency, infection and inflammation from disease, acute or chronic blood loss, and genetic Hb disorders (Sundararajan & Rabe, 2020), and is associated with debilitating symptoms, including fatigue, weakness, shortness of breath, dizziness, headaches and depression (van Haalen *et al*., 2020).

Thus, iron deficiency is the most common cause of anemia worldwide. Iron deficiency anemia (IDA) is a widespread public health problem, particularly in low- and middle-income countries. The WHO estimates globally that ~273 million young children under 5 years are anemic, among which ~50% are estimated to suffer from iron deficiency. There is a high demand for dietary iron during infancy and preschool years to support physical growth, rapid brain development, and early learning capacity (Sundararajan & Rabe, 2020).

Defining an abnormally low Hb concentration requires understanding how Hb naturally varies by age, sex, pregnancy status, genetic and environ- mental factors, and, potentially, race. Hb varies with age, most dramatically in the first months of life. In the newborn, normal Hb concentrations are between 17 and 21 g/L, their highest point during life. Hb concentration then decreases through the first 2-3 months of life before increasing again in childhood, and then levels off, throughout adulthood before declining again in older age (van Haalen *et al*., 2020). Sex differences in Hb concentrations begin in puberty (because of the effect of menstruation on iron stores and, subsequently, anemia) and continue throughout the reproductive years. During pregnancy, because of the expansion of blood volume and consequent dilution effect, Hb concentration naturally declines during the first sickle and second trimesters, rising gradually again in globin the third trimester. Apart from physiological factors, behavior and environmental conditions, such as altitude and smoking, can also affect Hb concentrations (Chaparro & Suchdev, 2019).

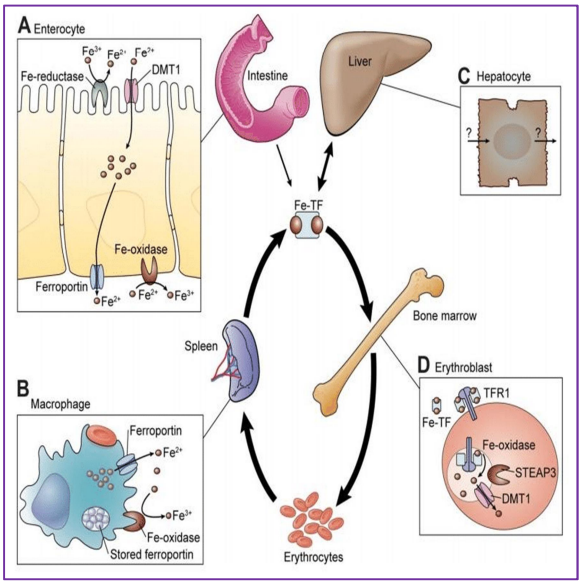


Figure 1 : Overview of iron homeostasis (Officioso, 2016).

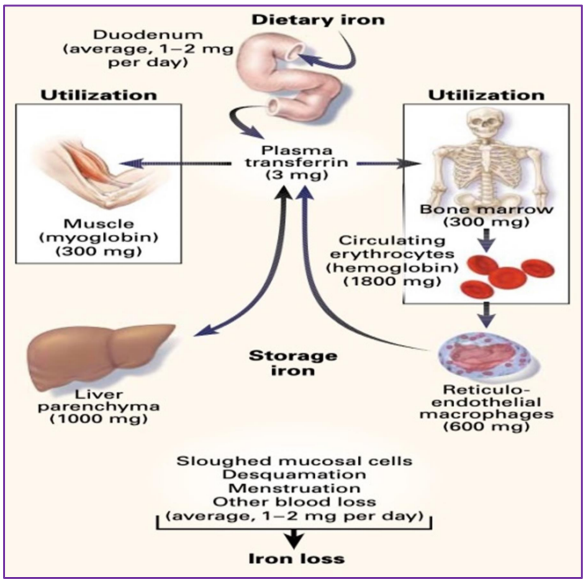


Figure 2 : Distribution of Iron in Adults (Salama, 2010).

2. Types and Classification of Anemia

Anemia is frequently classified based on the biological mechanism of causation (e.g., IDA, hemolytic anemia, and anemia of inflammation (AI)) and/or the RBC morphology (Chaparro & Suchdev, 2019).

There are several types and classifications of anemia. The occurrence of anemia is due to the various red cell defects such as production defect (aplastic anemia), maturation defect

(megaloblastic anemia), defects in hemoglobin synthesis (iron deficiency anemia), genetic defects of hemoglobin maturation (thalassemia) or due to the synthesis of abnormal hemoglobin (haemoglobinopathies, sickle cell anemia and thalassemia) and physical loss of red cells (hemolytic anemia) (Soundarya & Suganthi, 2016).

The classification of anemia based on two factors; Red cell morphology and Etiology of anemia (Table 2):

2.1.Anemia Classification Based on Morphology

Anemia can be classified based on morphology as:

- Normocytic normochromic (MCV 76–96 fL, MCHC 30–35 gm/dL): It is observed in acute blood loss, liver disease, endocrinopathy, anemia of infections, etc.
- Macrocytic (MCV >96, MCHC 30–35 gm/dL): It is observed in vitamin B12 and folic acid deficiency, etc.
- Microcytic (MCV <76 FL, MCHC 30 gm/dL): It is observed in iron deficiency anemia, thalassemia, sideroblastic anemia, pyridoxine deficiency, etc (Saxena *et al*., 2018).

2.2.Anemia Classification Based on Etiology

- Anemia due to blood loss :

– Acute loss: It may be external (e.g., as after trauma or obstetric hemorrhage) and internal [e.g., as bleeding from gastrointestinal (GI) tract, rupture of spleen, ruptured ectopic pregnancy, and subarachnoid hemorrhage].

– Chronic loss: It could be due to worm infestation, menses, repeated blood donation, repeated phlebotomy as treatment of polycythemiavera, etc.

- Hemolytic anemia due to destruction of red blood cells (RBCs)
- Impaired RBC production :

– Defective proliferation and differentiation of stem cells

- Aplastic anemia
- Chronic renal failure
- Endocrinopathy (defective production of hormones of pituitary, thyroid, suprarenal glands, testis)
- Defective proliferation and maturation of differentiation of the blasts:

– Defective deoxyribonucleic acid (DNA) synthesis: Vitamin B12, folic acid deficiency

– Defective Hb synthesis:

- Heme: Iron deficiency, pyridoxine deficiency
- Globin: Thalassemia and hemoglobinopathies
- Sideroblastic anemia
- Anemia of chronic disease: Infections, inflammation, neoplasms

– Myelophthisis due to infiltration of bone marrow (Saxena *et al*.,2018) .

Table 2 : Types and Classification of anemia (Saxena *et al*.,2018).

Types	Lab values	Causes
Macrocytic normochromic anemia	Increased MCV, normal MCHC MCV > 100 fL MCHC 34	Vitamin B12 deficiency Folate deficiency
Microcytic hypochromic anemia	Low MCHC Low MCV MCV < 80 fL MCHC < 30	Thalassemias; iron deficiency anemia; anemia of chronic disease (rare cases)
Normocytic normochromic anemia	Normal MCHC Normal MCV MCV > 80–99 fL MCHC 34	Anemia due to chronic disease, anemia of acute hemorrhage; aplastic anemias; hemolytic anemias

3. Common Causes of Anemia:

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). Furthermore, as anemia may have multiple causes, even in the same individual, hematological manifestations of a particular cause can be masked by another (Soundarya & Suganthi, 2016). 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. Although indices exist in clinical practice for distinguishing anemia etiology based on RBCs

parameters (e.g., IDA versus β-thalassemia—both cause hypochromia and microcytosis), their reliability for discriminating between causes varies (Chaparro & Suchdev, 2019).

A normal balanced diet will usually contain enough iron for your body's needs. The low dietary intake of iron, folic acid and food stuffs that promote iron absorption, coupled with poor bioavailability of iron are the major factor responsible for very high prevalence of anemia. Poor iron stores at birth, low iron content of breast milk and low dietary iron intake through infancy and childhood results in high prevalence of anemia in childhood (Soundarya & Suganthi, 2016).

As one of the most prevalent public health issues, anemia can result in major health issues such stunted growth in children, slowed mental and psychomotor development, worse work performance, and increased susceptibility to parasite infections. Low socioeconomic position, dietary deficits, helminth infections and other infectious illnesses, illiteracy, and blood disorders are the causes of anemia (Sundararajan & Rabe, 2020). However, Iron deficiency, folate deficiency, hookworm infection, and malaria are the main causes of anemia. About 50% of the two billion anemia cases worldwide are caused by iron deficiency (ID). This latter is believed to be the most common cause of anemia globally, but other nutritional deficiencies (including folate, vitamin B12, and magnesium) are also known to contribute to anemia (Hussien *et al*.,2023).

Among the other causes of anemia, heavy blood loss as a result of menstruation, or parasite infections such as hookworms, ascaris, and schistosomiasis can lower blood hemoglobin (Hb) concentrations. Acute and chronic infections, including malaria, cancer, tuberculosis, and HIV can also lower blood Hb concentrations. The presence of other micronutrient deficiencies, including vitamins A and B12, folate, riboflavin, and copper can increase the risk of anemia. Furthermore, the impact of hemoglobinopathies on anemia prevalence needs to be considered within some populations (Sundararajan & Rabe, 2020).

4. Global Burden of Anemia: Prevalence, Distribution, and Impact

Figure 1a–c depict the global estimates of the prevalence of anemia as a public health problem in infants and children aged 6–59 months, pregnant women aged 15–49 years, and in non-pregnant women aged 15–49 years, 2011. In 2011, population representative data sources from 107 countries worldwide revealed that 29% (496 million) of non-pregnant women and 38% (32.4 million) of pregnant women aged 15–49 years were reported to be anemic. Anemia

prevalence continues to be the highest in South Asia and Central and West Africa. Prevalence of IDA among infants and preschoolers living in rural India was 52.2% and 42.1%, respectively. In 2012, the World Health Assembly specified six global nutrition targets for 2025, with a commitment for a 50% reduction of anemia in women of reproductive age by 2025 compared to 2011 levels (Sundararajan & Rabe, 2020).

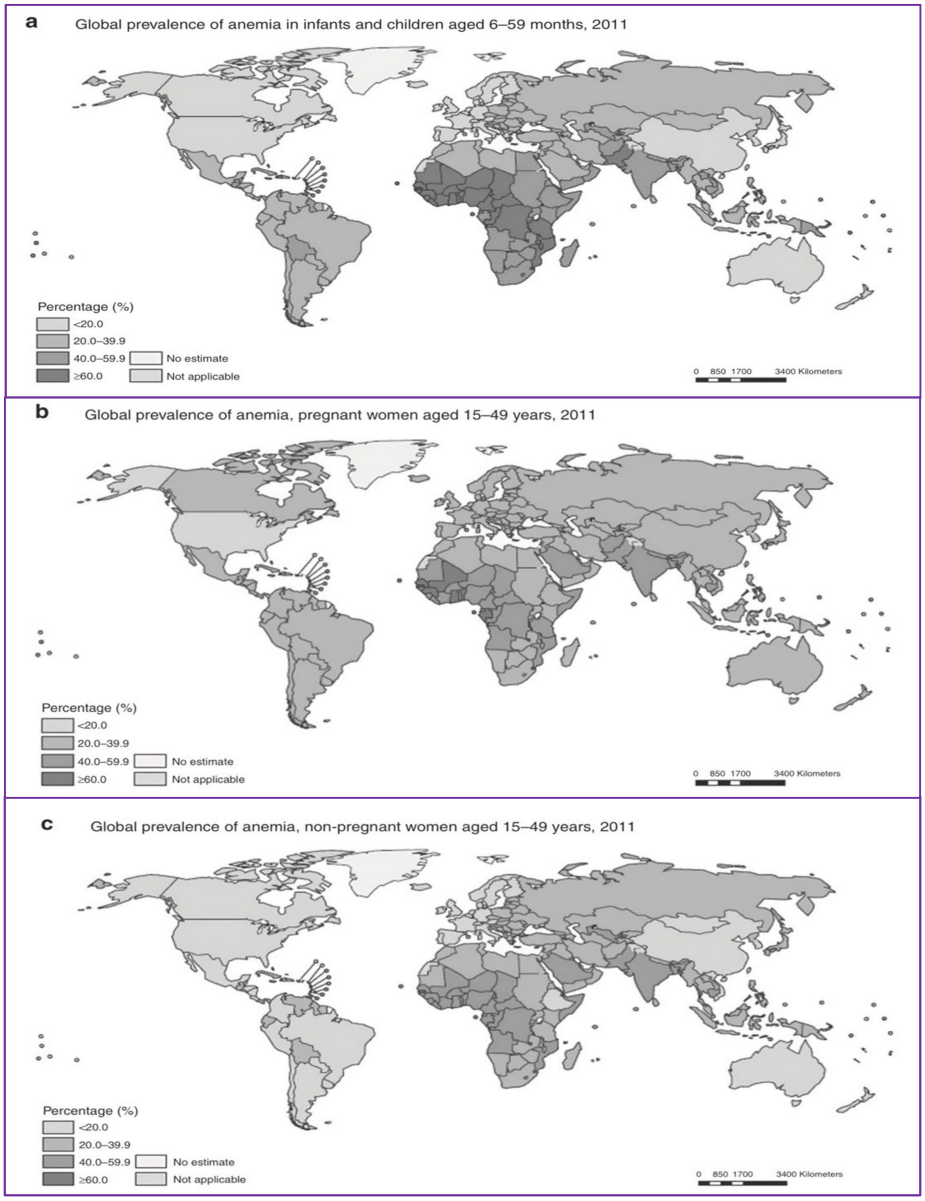


Figure 3 : Global prevalence of anemia (Sundararajan & Rabe, 2020).

As a global impact**,** the consequences of anemia can vary. It can affect school performance (through developmental delays and behavioral disturbances such as decreased motor activity, social interaction and attention to tasks), productivity in adult life and overall quality of life in general. During pregnancy, anemia has been associated with poor maternal and birth outcomes,

including premature birth, low birth weight and maternal mortality. In addition to the health consequences, anemia can have important financial impacts for individuals, families, communities and countries. It is estimated that for every US$ 1 invested in reducing anemia in women, US$ 12 in economic returns could potentially be produced (Lancet, 2019).

5. Anemia and oxidative stress

5.1. Oxidative stress

The global concept of "Oxidative Stress" is defined as "an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage" . It has developed from its initial formulation in 1985 to incorporate new knowledge on the role of redox signaling (Martemucci *et al*., 2022). The basic idea is that, in the open metabolic system, a steady-state redox balance is maintained at a given set point, which provides a basal redox tone, and that a deviation from the steady-state redox balance is considered a stress, initiating a stress response. Implicit in the definition of oxidative stress is (i) that a deviation to the opposite side of the balance is "reductive stress", and (ii) that there are physiological deviations, "oxidative eustress", and supraphysiological deviations, "oxidative distress" . Oxidative eustress is an essential part of redox control and physiological redox signaling (Sies, 2020).

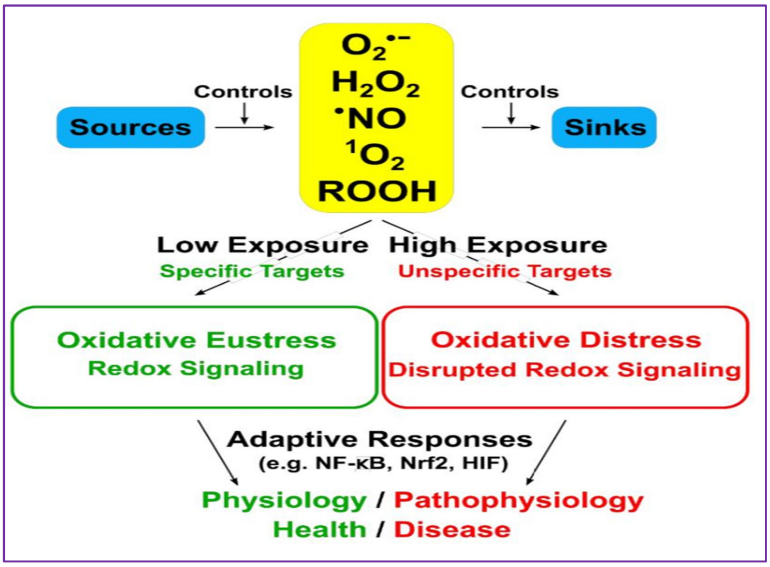


Figure 4: Oxidative stress and its relationship to redox signaling (Sies, 2020).

5.2. Free radicals

Free radicals are products of normal cell metabolism. When cells use oxygen, the redox process generates free radicals, normally ROS and RNS. They have acquired growing

importance in the fields of biology and medicine. They are produced during many different endogenous and exogenous processes. Most research on free radicals concerns oxygen radicals, which, together with some forms of active non-radical oxygen, are collectively called reactive oxygen species (Derouiche *et al*, 2022). In 1971, it was discovered that reactive oxygen species are generated in cell metabolic respiration; later, Halliwell and Gutteridge reported that ROS included free radical and non-radical derivatives of oxygen. In the 1980s, free radical nitric oxide (•NO) was identified in blood vessels and gave rise to studies on the biochemistry of reactive nitrogen species (RNS) (Martemucci *et al*., 2022).

In addition, free radicals can be defined as molecular entities or molecular fragments, capable of independent existence (hence "free"). They contain one or more unpaired electrons in an outer atomic orbital or molecular orbital (hence "radical"). The negative electrical charge of electron(s) may be counterbalanced by the positive nuclear charge of positrons, resulting in a neutral particle; otherwise, we have anion or cation radicals. The unpaired electron of a free radical is denoted by a point on the atom or group in which it predominantly resides, for example •H (hydrogen atom), •OH (hydroxyl), •CH3 (methyl) and •CH2CH3 (ethyl) (Sies, 2020). In oxygen radicals, the unpaired electron is located predominantly on an oxygen atom, e.g., superoxide (O2 •−), hydroxyl (•OH), peroxyl (ROO•). The odd number of electron(s) of a free radical makes it unstable, short-lived and highly reactive. This characteristic is responsible for chain reactions. Free radicals attempt to bond with other molecules, atoms or even individual electrons to create a stable compound. They either donate or accept an electron from other molecules, acting as oxidizing or reducing agents. The overproduction of free radicals can damage macromolecules such as nucleic acids (DNAs and RNAs), proteins and lipids. This leads to tissue damage in various chronic and degenerative diseases (Chetehouna *et al*., 2024).

5.3. Antioxidants

The term "antioxidants" in cellular defense against oxidants predominantly includes antioxidant enzymes with their substrates and coenzymes (Boulaares *et al.,* 2020).

The deleterious effects of excess free radicals, or oxidative stress, have been reported to eventually lead to cell death. The overproduction of reactive oxygen and nitrogen species has been implicated in the development of various chronic and degenerative diseases. The body has natural antioxidant defenses against free radicals. Antioxidants inhibit the oxidation process, even at relatively low concentrations, and can protect cells against free-radical damage by delaying or preventing the oxidation of proteins, carbohydrates, lipids and DNA. Antioxidants

have the ability to donate electrons. Antioxidants that break the chain reaction are strong electron donors and react with free radicals before major molecules are damaged. The antioxidants are therefore oxidized and must be regenerated or replaced. Antioxidant enzymes catalyze the degradation of species of free radicals, generally in the cell (Derouiche *et al.,* 2020).

Moreover, antioxidants can be endogenous or exogenous. The former may be enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GRx), or non-enzymes such as metabolic antioxidants, such as lipoic acid, glutathione, L-arginine, uric acid, bilirubin and antioxidant nutrients. Some exogenous antioxidant nutrients cannot be produced by the body and must be obtained from food; they include vitamin E, vitamin C, trace elements (Se, Cu, Zn, Mn) and phytochemicals such as isoflavones, polyphenols and flavonoids (Sies, 2020).

5.4. Relationship between anemia and oxidative stress

Anemia is closely correlated with oxidative stress, as erythrocytes represent a major antioxidant component of the blood (Abdel-Moneim *et al.,*2019). Oxidative stress and NO are known to modulate eryptosis in healthy red blood cells (RBCs) (Nader *et al*., 2020). RBCs contain exceptionally high levels of one type of protein, namely, hemoglobin (~98%) , which maximizes the capacity for oxygen transport by RBCs from the lungs to peripheral tissues. Carbonic anhydrase is the second most abundant protein and is responsible for the reversible conversion between carbon dioxide and bicarbonate ions. The partial pressure of oxygen is about 21% in the lung but rapidly decreases to <5% in peripheral tissues, and hence during blood circulation, RBCs are exposed to rapid changes in the oxygen environment . Reactive oxygen species (ROS) and/or reactive nitrogen oxide species (RNOS) are inevitably produced during the process of oxygen delivery and cause oxidative damage to RBCs (Fujii *et al*., 2021).

Eryptosis aims to prevent hemolysis of defective erythrocytes, but may lead to anemia, if it is not outweighed by a matching stimulation of erythropoiesis. Eryptosis may be induced by oxidative stress, which plays a crucial role in eryptosis-related pathophysiology in several diseases such as anemia (Bissinger *et al*., 2018).

Oxidative stress is a powerful stimulator of anemia and contributes to the development of anemia in a variety of clinical conditions. The stimulation of eryptosis may further compromise microcirculation, as eryptotic erythrocytes adhere to the vascular wall (Derouiche *et al*., 2018).

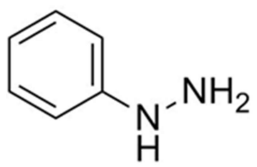
6. Experimental anemia

6.1. Defining phenylhydrazine

In 1895 Hermann Emil Fischer used phenylhydrazine (PHZ) for various reactions in sugars. PHZ exposure may cause red blood cell damage and in turn leads to anemia, it may also cause complications on the other tissues like spleen and liver. PHZ is proved to be mutagenic in vitro and known to exhibit genotoxicity *in-vivo* in rats. This Molecule is also found to induce acute hemolytic anemia in animal models. PHZ is one of the major intermediates used in the various industries for variety of purposes. Mainly it is used as a chemical intermediate in the agrochemical, chemical and pharmaceutical industries. Derivatives of PHZ are firstly used as antipyretics however due to its noxious action on red blood cells made their use vicious. PHZ used mainly for experimental purposes that is induction of anemia in rats (animals). PHZ shows a strong drug against polycythemia Vera a disorder, which will be characterized by rise in the total number of erythrocytes in the body (Shwetha *et al*., 2019).

- **Physicochemical Characteristics :**

Table 3: Physicochemical properties of phenylhydrazine (Shwetha *et al*., 2019).

Formula	C6H8N2
Boiling point	243.0±9.0ºc at 760mm Hg
Density	1.1±0.1g/cm Molecular
Weight	108.14g/mol
Monoisotopic Mol. Weight	108.068748266
Molecular Framework	Aromatic compounds
Vapor density	4.3(vs air)
Refractive index	n20/D 1.607
Color	Yellow to pale brown oily liquid
Structure	

6.2. Mechanisms of Phenylhydrazine-Induced Hemolysis

6.2.1. Alteration of iron metabolism

PHZ rises in the iron absorption, and produces the expression of iron transport genes [transferrin receptor (TFR1) and haemoxygenase (HO1)]. Haemoxygenase is a very important inducible enzyme involved in heme degradation, and also causes iron efflux from cell. Oral administration of PHZ at the dose of 40mg/kg/p.o for 7 days causes haemolytic anaemia in rats. Concentration of EPO was significantly increased by almost 5000-fold in the first couple of days followed by falling down to the basal level after 6days after PHZ infusion (de Oliveira et al., 2022). The mRNA expression of erythroferrone (ERFE), inhibits the production of hepcidin in the liver, and it helps in the synthesis of haemoglobin as this protein increases the amount of iron, was rapidly increased within the bone marrow and spleen 3 days after injection of PHZ and then gradually decreased but was still more than baseline on 6th day (Coffey and Ganz, 2018). Hepcidin, a regulator of iron metabolism, mRNA level was also found to be reduced by more than 8 times the basal level on 5thday. Mechanistic examination manifested that the increase of serum EPO essentially determined the induction of ERFE expression particularly at the primary 3 days after PHZ treatment. Hepcidin suppression is restrained significantly by ERFE overthrow which is mediated by Lentiviral elements under PHZ treatment. Thus, EPO dependent ERFE expression acts as an erythropoiesis-driven regulator of iron metabolism under PHZ-induced haemolytic anaemia (Shwetha *et al*, 2019).

6.2.2. Haemolytic anaemia

PHZ causes haemolytic anaemia to know about EPO, pathological, regenerative response through clinical and morphological studies. PHZ is given through oral, inhalation and dermal routs that causes oxidative stress within erythrocytes which results in oxidation of oxyhemoglobin leads to the formation of methemoglobin and later converts into irreversible hemichromes which causes precipitation of haemoglobin in the formation of Heinz bodies (Orrico *et al.,* 2023). PHZ causes impairment in skeletal protein, lipid peroxidation, ATP depletion, imbalance of cation and decreased membrane deformability. All these symptoms figure outs haemolytic anaemia (Shwetha *et al*., 2019).

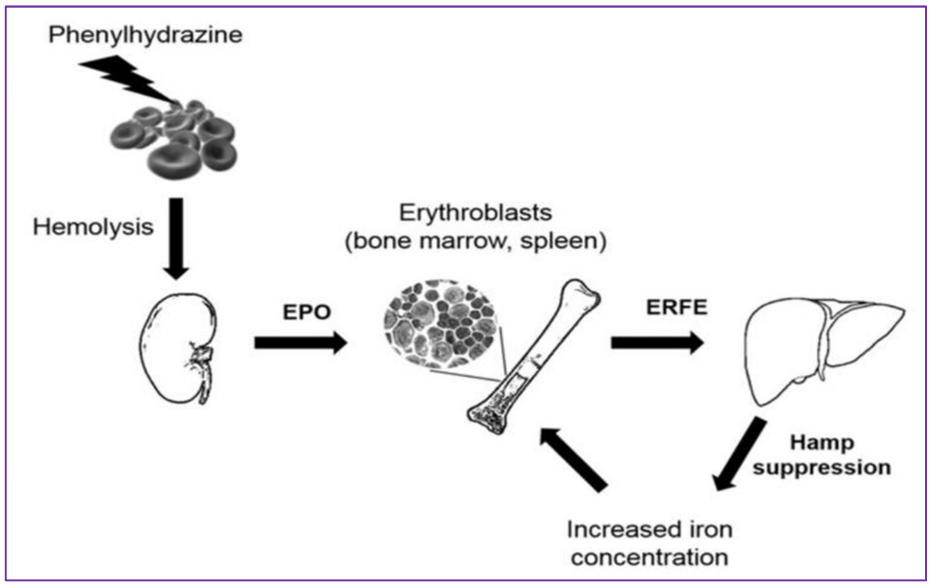


Figure 5 : Mechanism of PHZ induced anaemia (EPO -erythropoietin, ERFE-erythroferrone, Hamp-Hepcidinantimicrobial peptide) (Shwetha *et al.*, 2019).

Chapter II

Camel milk & Zinc nanoparticles

1. Camel Milk *(Camelus dromedaries)*

Dromedary Camel milk (CM), scientifically called *(Camelus dromedarius)*, is a widely consumed beverage in African and Arab countries, particularly in the Sahara areas of Algeria, due to its medicinal and dietary benefits. It is believed to have the potential to promote functional health (Amrouche *et al*., 2020), and has therapeutic and antimicrobial properties. It is also considered to have anti-cancer and anti-diabetic effects. Despite its various health benefits, pasteurized CM is only available in a few countries, such as Mauritania and Algeria (Zahra *et al*., 2021).

1.1. Chemical Composition of Camel Milk

Camel's milk is typically white and opaque in appearance, with a taste that can range from sweet and sharp to salty depending on the animal's diet and water availability. Its pH level ranges from 6.2 to 6.5, and its density ranges from 1.026 to 1.035. Compared to cow's milk, CM sours slowly and can be stored for longer periods without refrigeration. A study of the chemical composition and nutritional quality of CM revealed that it contains 11.7% total solids, 3.0% protein, 3.6% fat, 0.8% ash, 4.4% lactose, 0.13% acidity, and has a pH of 6.5. CM has higher levels of Na, K, Zn, Fe, Cu, Mn, niacin, and vitamin C but relatively lower levels of thiamin, riboflavin, folacin, vitamin B12, pantothenic acid, vitamin A, lysine, and tryptophan compared to cow's milk (Fufa & Haile, 2020).

1.2. Protein composition of camel milk

Proteins are regarded as the primary component of milk, significantly influencing its nutritional value due to their diverse composition and properties, including biological, technological, and functional aspects. According to (Benmeziane, 2021), the protein content of camel milk ranges from 2.15% to 4.61%, divided into two main groups: casein and whey proteins.

Caseins are the most abundant proteins in camel milk, comprising between 52% and 87% of the total protein content, as indicated by (Singh *et al.*, 2017).There are notable differences between the caseins of camel milk and those of other ruminants' milk. Specifically, camel milk caseins include the three fractions (αS1-, αS2-, and β-caseins). It has been observed that these fractions are very similar to those in cow’s milk, with the exception of κ-casein, which represents 13.6% in cow's milk but only 5% in camel milk (Benmeziane, 2021).

Whey proteins are the second major class of proteins in camel milk, constituting 20% to 25%

of the total milk proteins. The distribution of whey proteins in camel milk differs from that of other milks. In this context, α-lactalbumin is the major whey protein, existing in two different forms with identical molecular weights (Jilo, 2016). Other whey proteins found in camel milk include serum albumin, lactoferrin, proteose peptone component 3 (PP3), and peptidoglycan recognition protein (PGRP). Additionally, immunoglobulins IgG2 and IgG3 are present (Benmeziane, 2021).

1.3. Health Benefits Properties of Camel Milk

Scientists have conducted research that proves the medicinal properties of CM.

1.3.1. Diabetes Control – a Potential Therapy

In diabetic patients, the sugar level in the blood increases due to the insufficient production of insulin by the pancreas. Recent research has shown that CM can reduce 55% of the blood glucose level. Type 1 diabetic patients can also reduce their insulin requirements by 30% by consuming CM. This is because CM contains proteins that are similar to human insulin (Akbar *et al*., 2020). Additionally, camel insulin is easily absorbed into the bloodstream and is encapsulated in nanoparticles, which allows it to pass through the stomach and enter circulation (Fufa & Haile, 2020).

1.3.2. Camel milk as Anti-hyperlipidemic Agent:

The levels of certain blood components like lipids, lipoproteins, and triglycerides can vary in people with diabetes, which can lead to complications in the blood vessels and increase the risk of heart failure. Recent studies have shown that treating type 1 diabetes patients with CM for 6 months can lead to a reduction in the levels of Low-density lipoprotein. In addition, a three-fold reduction in lipid profile was observed in type 1 diabetic patients who were given CM for a period of three months (Akbar *et al*., 2020).

1.3.3. Anemia:

Soni *et al*., (2021) have illustrated the impact of CM on the prevalence of anemia, revealing a significantly lower incidence among consumers of camel milk compared to non-consumers. This outcome can be attributed to the high levels of Vitamin C, iron, zinc, and protein found in camel milk, which contribute to its nutritional benefits.. These data have been also obtained by (Abdurahman & Gashu, 2021).

1.4. Therapeutic and Nutritional Features of Camel Milk in Children

1.4.1. Diarrhea :

Frequent bowel movements, commonly known as diarrhea, can be caused by viruses, particularly Rota virus. A remedy for this condition is CM, which has been found to be effective in treating diarrhea in rats due to its high levels of potassium and sodium content (Akbar *et al*., 2020).

1.4.2. Autism :

Autism spectrum disorder is a condition that affects neurodevelopment and causes social orientation difficulties, communication impairments, and repetitive behaviors. According to research, clinical symptoms of autism have been observed to decrease in patients who consumed CM for two weeks. The positive effects were observed in patients above 21 years of age and in children under 15 years of age (Lord *et al*., 2018).

1.4.3. Lactose Intolerance:

Research studies have revealed that CM contains a lower amount of lactose in comparison to cow's milk. As a result, a group of 25 patients suffering from lactose intolerance were examined to determine the effects of consuming CM. The findings showed that the patients who had experienced indigestion after consuming cow's milk were able to tolerate and digest CM without any issues (Seidita *et al*., 2023).

1.5. Nutrient Absorption and Bioavailability:

1.5.1. Factors Affecting Zinc Bioavailability in Camel Milk:

- **Lactoferrin :**

CM contains a protein called lactoferrin that has the ability to attach to zinc and safeguard it from other dietary components that may hinder its absorption in the intestine, which could result in an increased absorption of zinc (Izadi *et al*., 2019).

- **Caseinophosphopeptides (CPP) :**

Just as with other types of milk, the casein phosphopeptides present in CM have the ability to create soluble complexes with zinc, which can enhance its solubility and bioavailability (Izadi *et al*., 2019).

- **Solubility and Stability :**

The bioavailability of zinc in CM can be affected by its solubility and stability. During digestion, zinc solubility and its capacity to remain in a bioavailable form may be influenced by factors such as pH, temperature, and the presence of other ions. These findings were reported by Amini *et al*., (2021).

- **Interaction with Other Nutrients :**

Certain nutrients found in CM, like proteins, fats, and carbohydrates, can have an impact on the absorption of zinc. For instance, phytates which are present in CM may attach themselves to zinc and lower its bioavailability. On the other hand, vitamin C may increase the absorption of zinc (Amini *et al*., 2021).

2. Nanotechnology

2.1. Definition:

Nanotechnology involves the production of particles that are between 1 to 100 nm in size by using various synthesis methods, as well as modifying their structure and size. The use of nanoparticles has become increasingly prevalent across a variety of fields, including molecular biology, physics, organic and inorganic chemistry, medicine, and material science. When particles are reduced to nano-size, they exhibit unique and improved properties, such as particle size distribution and morphology, that are not seen in larger particles of bulk material (Jamkhande *et al*., 2019).

2.2. Nanoparticles :

Spherical particles called nanoparticles are composed of either natural or artificial polymers and can range in size from 10 to 500 nm. Due to their high surface area-to-volume ratio and spherical shape, these particles have a variety of potential applications. Synthetic nanoparticles come in the form of either metal nanoparticles, such as gold and silver nanoparticles, or metal oxide nanoparticles, including zinc oxide (ZnNPs) (Rahim *et al*., 2018;).

2.3. Zinc Oxide Nanoparticles (ZnONPs) :

Zinc oxide is an inorganic chemical compound widely used in everyday life. Noticeable

in recent years, the very rapid development of nanotechnology has led to the development of zinc oxide nanomaterials with new properties (Czyżowska & Barbasz, 2020). ZnNPs, which are a dietary supplement and food additive, are frequently used in food for medicinal purposes. They have also been applied to food packaging materials. Additionally, ZnNPs are commonly used in the medical field, particularly in dental implants, because of their exceptional antibacterial and antifungal properties (Dkhil *et al*., 2020). Zinc oxide has photocatalytic properties; however, its stability is not very good. Zinc oxide nanoparticles exhibit greater stability, moreover, better crystallinity and smaller defects, so they can be effectively used in the process of degradation of various substances like organic impurities **(**Chetehouna *et al.,* 2020).

2.4. Synthesize of nanometals :

In recent years, nanoparticle synthesis has witnessed a remarkable shift toward sustainable and environmentally friendly approaches. Conventional nanoparticle synthesis methods frequently involve using hazardous chemicals and high-energy processes, raising environmental concerns, and producing toxic by-products. On the other hand, green synthesis methods provide a viable solution by utilizing bio-based materials .This method is used in several fields as shown in (Figure 6) (Osman *et al*., 2024).

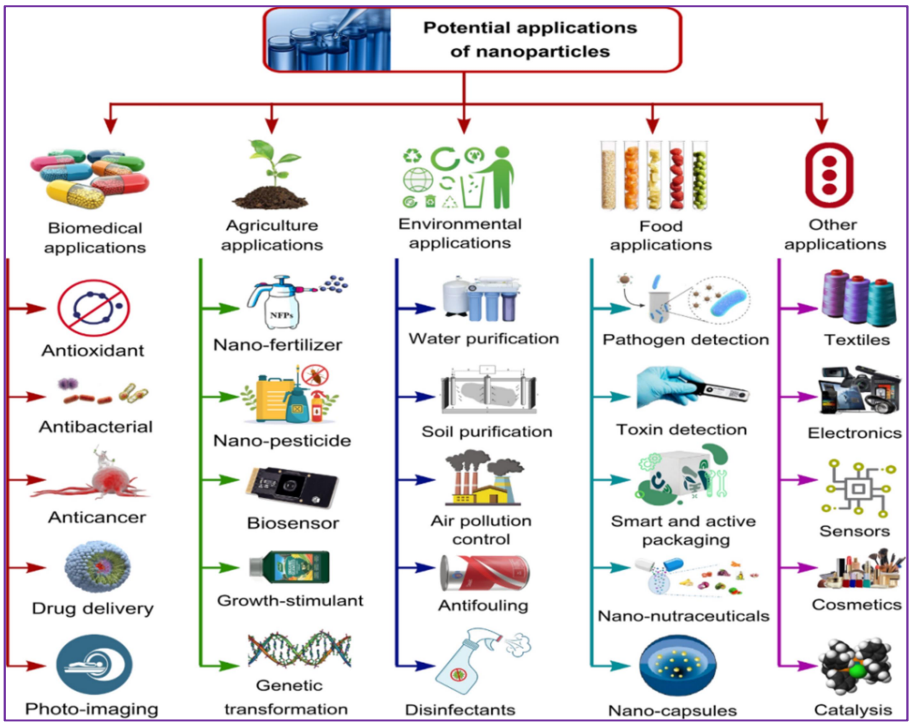


Figure 6 : Applications of nanomaterials in various sectors (Osman *et al*.,2024).

2.5. Green synthesis:

It is a process that involves constructing nanomaterials in a clean, safe, cost-effective, and environmentally friendly manner. The final morphology and size of the nanoparticle are determined by different active molecules and precursors, such as metal salt. The green species utilized in the synthesis of nanomaterials often consists of specific enzymes, amino acid groups, proteins, or chemical structure (Dkhil *et al*., 2020).

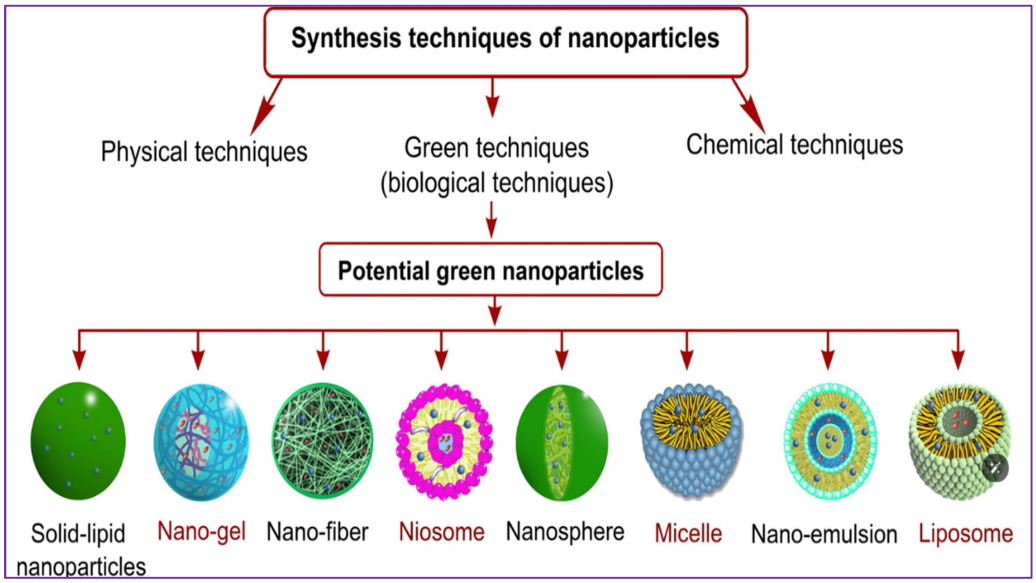


Figure 7 : Nanoparticle synthesis (Osman *et al*., 2024).

Green nanoparticles offer broader utility and innovation potential, spanning fields like nanoelectronics, nanosorbents, and catalysis, as shown in (Figure 7) (Derouiche *et al*.,2022).

2.6. The differences between traditional and green nanosynthesis methods:

Nanomaterial synthesis can be subdivided into two main categories: traditional methods and green methods. Their differences are shown in (Table 4) (Dkhil *et al*., 2020).

Table 4 : The distinguished point between Traditional & Green nanosynthesis (Dkhil *et al*., 2020).

Aspect	Traditional Nanosynthesis	Green Nanosynthesis
Variety of Nanoparticles	Produces a large variety of nanoparticles with vast applications	May produce a narrower range of nanoparticles with limited applications
Scalability	Some methods offer extensive scalability	Scalability may vary depending on the method and resources available
Control over Morphology	High control over nanoparticle morphology	Control over morphology may be more limited
Applications	Used in innovative battery conduction, electrical applications, targeted disease therapy, energy storage/conservation	Applications may focus on sustainable technologies, biocompatible materials
Environmental Impact	Utilization of organic solvents poses neurobehavioral and reproductive risks; high pressure and heat conditions may create dangerous working conditions; concerns for volatile vapor and excessive carbon dioxide production	Designed to adhere to the 12 Principles of Green Chemistry, minimizing environmental impact and health risks
Overall Risk	Potential irreversible risks to both scientists and the environment	Aimed at minimizing risks to human health and the environment

Second Part

Experimental Part

Chapter I

Materials & Methods

I. Materials

1. Biological materials

1.1. Sampling of camel milk :

The milk samples utilized are sourced from healthy herds of *(Camelus dromaderius)* dromedary camels belonging to the Arbia population, raised in (Mihaouenssa), El-Oued, Algeria during the month of October, 2023.

1.2. Extraction of milk proteins :

Sample collection was carried out in the morning and were immediately frozen at -20°C without preservatives. The steps are explained in the following (Figure 8) (Saliha *et al*., 2013).

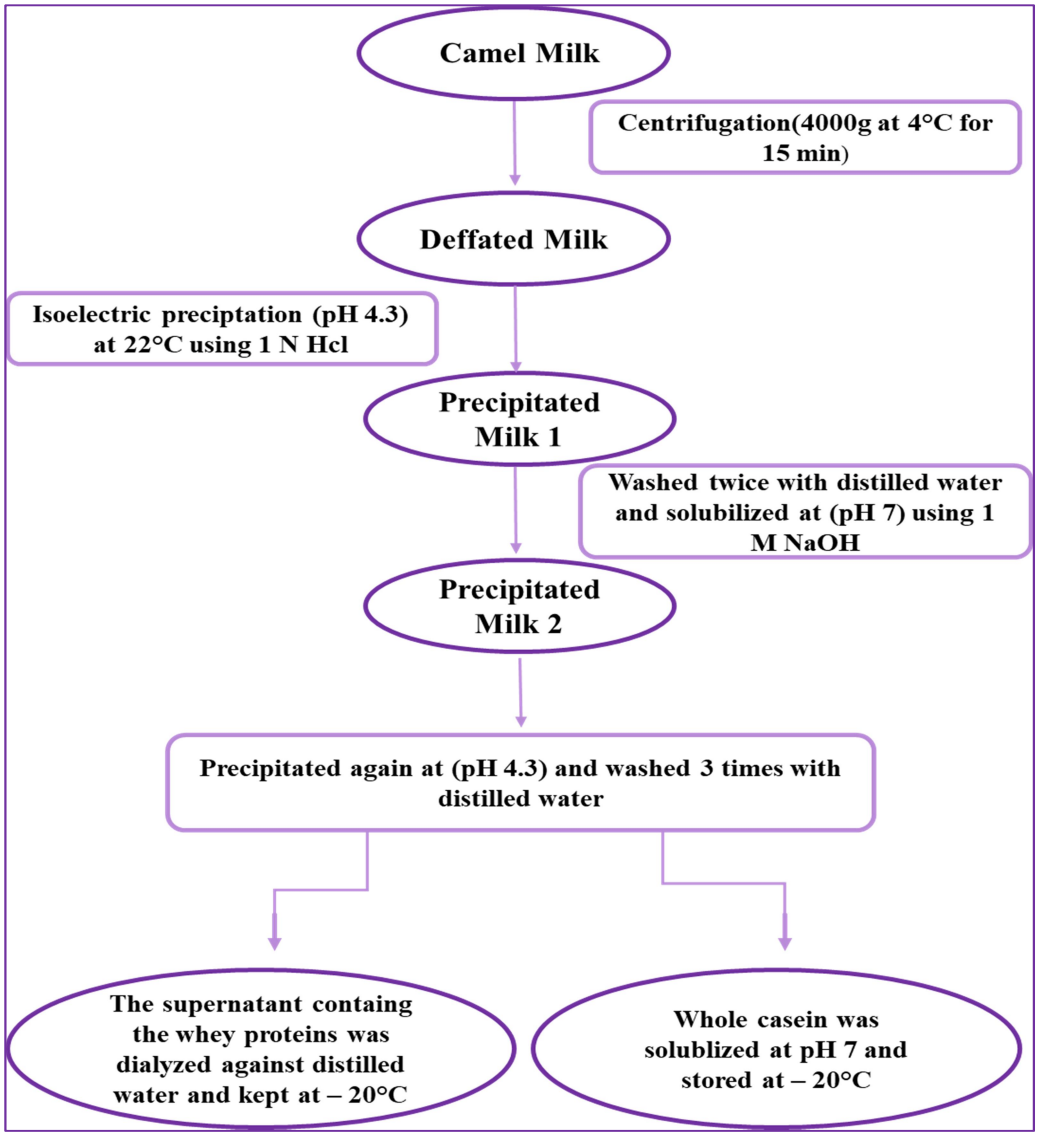


Figure 8 : Preparation of milk sample

2. Animals

2.1 Animals' care :

In this study, Twenty males *Albino Wistar* rats at the age of 8 weeks old, weighting 172.7±7.175 g, obtained from Pasteur Institute in Algiers. Rats were given a free access to their standard diet and tap water during the study. The animals were carried under the same conditions of and an ambient temperature. All experimental procedures employed, as well as rats' care and handling, were in accordance with guidelines provided by the local ethics. After a period of adaptation, the animals were divided into four experimental groups. The experiment was conducted over a period of 20 days.

2.2. Experimental Design :

In order to conduct this study, a group of twenty male *Albino Wistar* rats were divided into four weight equaled groups with five rats in each. After a period of adaptation , the injection process was started . The rats had to fast for 12h before their injections . All in the same day , control group was injected with distilled water and the three remained groups were injected with phenylhydrazine (40mg/Kg) through intraperitoneal injection. The process was repeated the day after with the same conditions. Afterward, the treatment injections started. Control and PHZ group were injected with only distilled water. As for the rest , group N°3 was injected with CP-ZnNPs (7mg/Kg) and group N°4 with WP-ZnNPs (7mg/Kg). This very last process was repeated once a week for three times (Okafor & Atsu, 2022).

→ **Group 1 (Control group):** Healthy rats, intraperitoneal injection with physiological water.

→ **Group 2 (PHZ group):** PHZ Anemia rats received distilled water (40mg/Kg).

→ **Group 3 (CP-ZnNPs group):** PHZ Anemia rats treated by CP-ZnNPs through intraperitoneal injection (7mg/Kg).

→ **Group 4 (WP-ZnNPs group)**: PHZ Anemia rates treated by WP-ZnNPs through intraperitoneal injection (7mg/Kg).

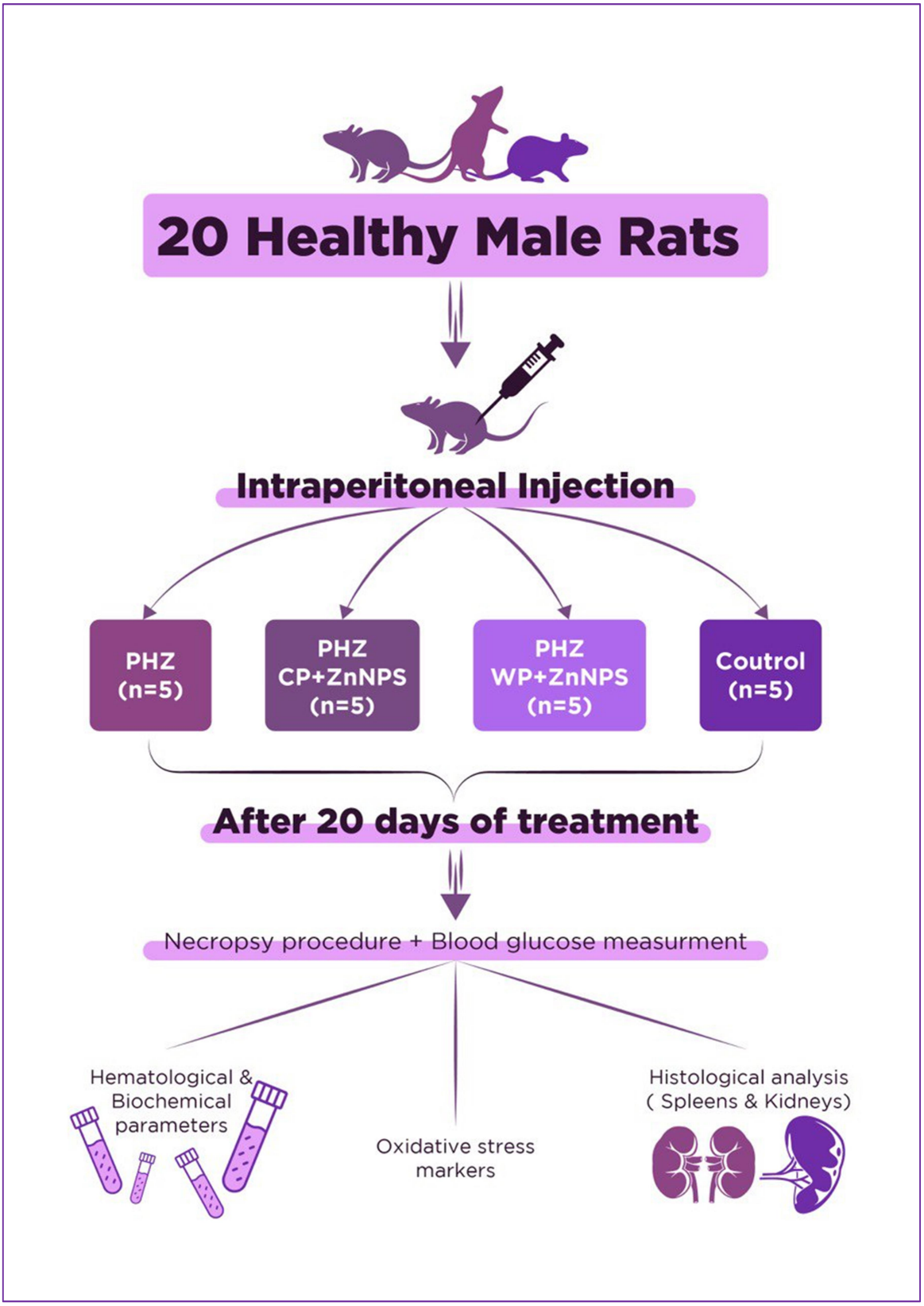


Figure 9 : Experimental design of the study.

3. Necropsy procedure

After 12 hours of fasting, these rats were sacrificed using scalpels after inhaling chloroform as anesthesia. At first, Glycemia level was measured during rat's sacrifice using glucometer. In addition to collecting blood samples into EDTA tubes to carried FNS and dry tubes, the serum was obtained by centrifugation for 10 min at 3000 tour/min to be used for biochemical analysis assays. The dissection of the dead rats and macroscopic examination along with the collection of organs; Liver, Spleen, Heart, Testicles and Kidneys, was the last step. After washing these organs with 0.9% saline organs were stored at -20 C for oxidative stress tests. As for histological analysis, pieces of Spleen and Kidneys were collected from each group into 10% formaldehyde.

II. Methods

1. *In-vitro* study

1.1. Biosynthesis of immobilized protein by zinc oxide ZnO nanocomposites

In order to synthesize the ZnO nanoparticles, 2.00 g protein was dissolved in 25 mL distilled water. After then 7.04 g Zn (NO3)2.6H2O is added to 200ml distilled water. protein solution is added drop wise to Zn-salt solution with continuous stirring. The solution was kept for overnight for precipitation. Precipitation was then centrifuged three times at 6000 rpm for 25 min to ensure the complete separation of nanoparticles. Centrifuged nanoparticles were then air dried in an oven at 50 °C for three hours and washed in double-distilled water and ethanol, respectively (Chetehouna *et al*., 2020).

1.2. Characterization of zinc nanocomposites

The characterization of ZnO was identified by Ultraviolet-visible spectroscopy (UV-VIS), Fourier transform infrared spectroscopy (FTIR),Scanning electron microscope (SEM) and energy dispersive X-ray (EDX).The analysis were done by direct reading through JENWEY, Cary 630 FTIR and Phenom Prox, respectively.

1.3. Determination of Protiens

→ **Principle**

The tissue proteins were determined according to a colorimetric method by a SHIMATZU type spectrophotometer using Coomassie blue as a reagent, which is reacted with the amine group (NH2) of the proteins to form a blue complex. The appearance of the blue color reflects the degree of ionization of the acid medium and the intensity corresponds to the concentration of proteins. The absorption is measured at 595 nm (Bradford, 1976).

→ **Preparation of Bradford's reagent**

-Dissolve 100 mg of Coomassie blue in 50 ml of ethanol (95%) .

-Shake the mixture for 2 hours with a shaker away from light .

-Add 100 ml of orthophosphoric acid (H3PO4) (85%) .

-Complete the volume to 1 liter with distilled water .

-Filter the solution obtained with filter paper .

Note: This reagent is stable for 2 weeks at 4°C.

→ **Procedure**

l. Take 01 ml of the protein solution .

2. Add 5 ml of Coomassie blue.

3. Shake and let to stand for 5 minutes.

4. Read the optical densities against the blank at 595 nm.

5. Compare the protein concentration in the tissues studied.

The protein concentration is determined by comparison with a standard range of bovine serum albumin (0.1-0.2-0.4-0.6-0.8-1mg / ml) previously carried out under the same conditions.

1.4. Biological activities

1.4.1. Antioxidant activity (1,1-diphenyl-2-picrylhydrazyl "DPPH assay")

The in vitro antioxidant activity was assessed by measuring the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical's scavenging capacity. A spectroscopic method was used to determine the relative antioxidant ability of extracts through a DPPH radical scavenging assay. Following the method described by Nwidu *et al.,* (2017) stock solutions of the extracts (5 mg/ml) were prepared and diluted to concentrations of 200, 100, 50, 25, 12.5, and 6.25 µg/ml in ethanol. To this, 160 µL of 0.1 mM DPPH in ethanol solution was added to 20 µL of the extracts or standard, followed by mixing with 20 µL of H2O. B-tocopherol was used as a control solution across a concentration range of 1.56, 0.78, 0.39, 0.195, and 0.0975 mg/ml, assayed under similar conditions. The mixtures were then incubated at 37°C for 40 minutes in the dark, and the absorbance of the samples was measured at 517 nm, as outlined in Nwidu *et al*., (2017).

$$\textbf{\textit{Inibition equation}}\ (\%) = \frac{\textbf{\textit{OD Control}} - \textbf{\textit{OD Sample}}}{\textbf{\textit{OD Control}}} \times \mathbf{100}$$

1.4.2. Anti-inflammatory activity (Hemolysis assay)

Hemolysis assay was done as described by study of vinjamuri *et al*., (2015). 5mL of blood was collected from healthy volunteers in the tubes containing 5.4 mg of EDTA to prevent coagulation and centrifuged at 1000 rpm for 10 min at 40 °C. Plasma was removed carefully and the white buffy layer was completely removed by aspiration with a pipette with utmost care. The erythrocytes were then washed for additional three times with 1X PBS, pH

7.4 for 5min. Washed erythrocytes were stored at 40C and used within 6 h for the hemolysis assay. 50 uL of 10 erythrocytes were stored at 40C and used within 6 h for the hemolysis assay. 50 uL of 10 dilutions (100 uL. Erythrocytes suspension: 900 L 1XPBS) of erythrocytes suspension was mixed with 100 uL of test samples (48g/mL), 100 uL of IXPBS was used active control and 100 uL of 1% SDS as positive controls. Reaction mixture was incubated at 37°C water bath for 60 min. Volume of reaction mixture was made up to 1 mL by adding 850 pL of IXPB. Finally, it was centrifuged at 300rpm for 3min and the resulting hemoglobin in supernatant was measured at 560 nm by spectrophotometer to determine the concentration of hemoglobin. Percentage haemolysis was calculated as follows:

$$\textbf{\textit{Hemolysis inhibition}}\ (\%) = \mathbf{100} - \frac{\boldsymbol{OD\ sample}}{\boldsymbol{OD\ control}} \times \mathbf{100}$$

2. *In-vivo* study

2.1. Biochemical parameters

Determinations of biochemical parameters : triglyceride (TG), total cholesterol (TC), Fasting blood glucose, Aspartate aminotransferase (ASAT), Alanine aminotransferase (ALAT), Plasma urea, Plasma creatinine, Serum iron, Lactate dehydrogenase (LDH) were done using commercial kits by the automatic analyzer (MINDRAY Bs 240).

2.2. Hematological parameters

The hematological parameters (FNS) was performed by the hematology auto analyzer (SYSMEX).

2.3. Oxidative stress parameters

2.3.1. Homogenates preparation

Approximately 1g of liver, kidneys, spleen, testicles, and heart tissues was homogenized in 9ml of Tris-buffered saline (TBS, pH 7.4). The homogenates were then centrifuged at 3900 rpm for 20 minutes, and the resulting supernatant was utilized to determine oxidative stress parameters (Chetehouna *et al*, 2024).

2.3.2. Determination of malondialdehyde (MDA) level

MDA levels were assessed using the method outlined by SASTRE *et al.* (2000). Briefly, pipette 300 µl of the sample and 1200 µl of TBA reagent into glass test tubes, seal them, and heat the mixture in a boiling water bath at 100 °C for 15 minutes. After heating, cool the tubes

in a cold water bath for 30 minutes with the tubes open to release gases produced during the reaction. Centrifuge at 3000 rpm for 5 minutes and measure the absorbance of the supernatant at 532 nm with a spectrophotometer. The TBARS concentration was calculated using the MDA molecular extinction coefficient (ε= 1.53 x 10^5 M^-1 cm^-1), and the results were expressed in µmol/mg protein.

2.3.3. Determination of Super Oxide Dismutase (SOD) activity

→ **Procedure**

The assay method for SOD activity involves using NBT by the superoxide anion (O2.-) as a basis for detecting the presence of SOD by measuring the absorbance spectrophotometrically at 560 nm (Beauchamp & Fridovich, 1971).

Collect in tubes	Blank	Sample
EDTA-Met (0.1mM, 13mM)	1000µL	1000µL
Phosphate buffer (50Mm)	892,2µL	892,2µL
Sample	-	50
Phosphate buffer (50Mm)	1000µL	950µl
NBT (75µM)	85,2µL	85,2µL
Riboflavin (2µM)	22,6µL	22,6µL

→ **Expression of results**

Inhibition percentage of NBT reduction by SOD

$$\text{IP\%} = \frac{OD\ blanc - \text{OD sample}}{OD\ blanc} \times 100$$

50% (IP) =1UI/L of SOD

2.3.4. Determination of reduced glutathione (GSH) level

→ **Principal**

The level of reduced glutathione is determined according to (Weckbecker & Cory, 1988) by measuring the optical density results from the formation of 2-nitro-5-mercocapturic

acid (TNB) from the reduction of dithio-bis-2-nitrobenzoic acid (DTNB) ,which is called Ellman reagent with SH groups exist in GSH briefly.

→ **Procedure**

1. 800µl of homogenate samples are add to 200µl of salicylic acid (0.25%).
2. The mixture was centrifuge at 1000 rpm for 5 min.
3. Take 500 ml of supernatant and mixed with 1000µl of tris buffer (tris 0.4mol, 0.02mol NaCl, ph = 8.9) and 25 µl of DTNB (0.01 mol/L).
4. Read the absorbance at λ= 412 nm after 5 min of incubation.

→ **Expression of results**

The calculate of GSH concentration expressed in nanomoles per milligram of proteins (nmol/mg of prot) according to the following formula:

$$\boldsymbol{GSH(nmol/mg\ of\ prot) = \frac{(OD \times 1 \times 1.525)}{13133 \times 0.8 \times 0.5 \times mg\ of\ prot} \times d}$$

- **OD:** Optical Density.
- **1.525:** total volume of blend an ml.
- **13133:** Absorption constant of SH groups at 412 nm.
- **0.5:** volume of solution float an ml.
- **1:** volume of protein mixture.
- **0.8:** volume of homogeneous solution without protein exists in 1 ml.
- **GSH:** concentration of glutathione.

2.3.5. Determination of Catalase (CAT) activity

In test tubes, briefly mix 1 ml of phosphate buffer (0.1M, pH 7.2), 0.975 ml of freshly prepared H2O2 (0.091M), and 0.025 ml of the enzyme source (serum). Measure the absorption at 560 nm every minute for 2 minutes (Aebi. H, 1984).

$$\boldsymbol{CAT\ (UI\ /g)\ = \frac{(2,3033\ /T)}{g\ of\ pr} x\ (\ log\ A1\ /\ log\ A2\ )}$$

2.4. Histopathology examination of spleen and kidneys tissues

After decapitation by cervical dislocation and dissection, spleen and kidneys organs were rapidly dissected out from each animal, washed, and fixed in a formaldehyde solution (36%) for a while untile slices preparation. Fixed samples from spleen and kidneys were washed, dehydrated in an ascending series of ethanol, cleared with toluene, embedded in paraffin blocks and sectioned into 4–5µm sections using Microtome (Thermo scientific) for serial specimens, then mounted on glass slides and stained with hematoxylin and eosin (H & E) stain (Banchroft *et al*.,1996). The slides were observed under an histological light microscope (ZEISS) at 10X and 40X magnification.

2.5. Statistical analysis

The obtained values were represented as mean ± standard error mean (SEM). The statistical evaluation is carried out using the student's T-test for comparison between our experimental groups. All data in this study were examined by Minitab 13.0 software. $P < 0.05$ indicates a statistically significant difference.

Chapter II

Results & Discussion

I. Results

1. *In-vitro* assays of zinc oxide nanoparticles (ZnONPs)

1.1. Characterization of ZnONPs

1.1.1. UV–Vis spectroscopy

Figure 10 showed the presence of ZnONPs which confirmed by obtaining a spectrum in the visible range of 200 nm-400 nm using UV-visible spectrophotometer. This analysis revealed that the absorbance peak for ZnONPs is specifically located at around 300 nm.

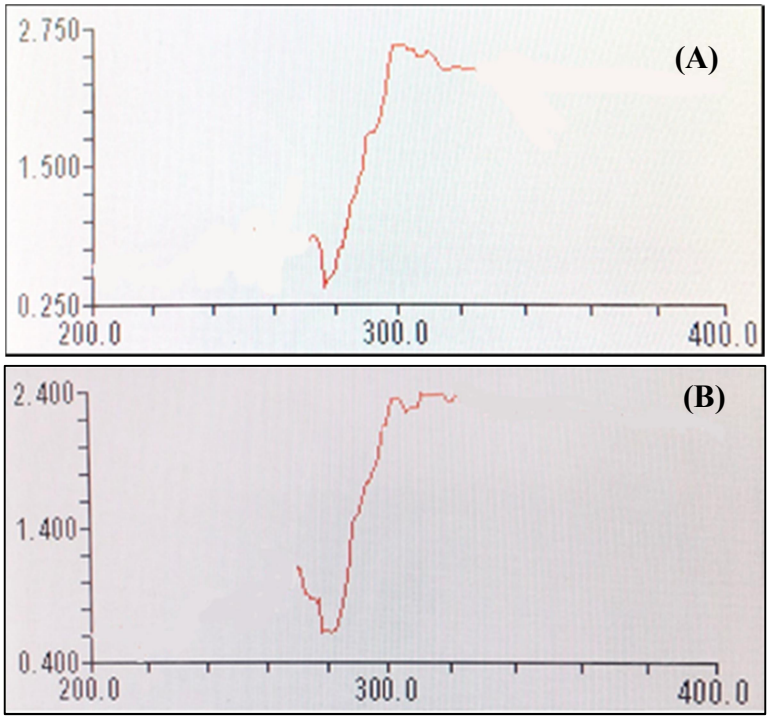


Figure 10: UV-Vis spectrum of Zinc nanoparticles using casein milk protine **(A)** and whey milk protine **(B) (Original photo)**

1.1.2. FT-IR analysis

IR absorption spectrums of ZnONPs formuleted by casein milk protine and whey milk protine are shown in (Figure 11), it was observed that the presence peak between 400-700 cm^{-1} corresponds to the O-Zn, compound.

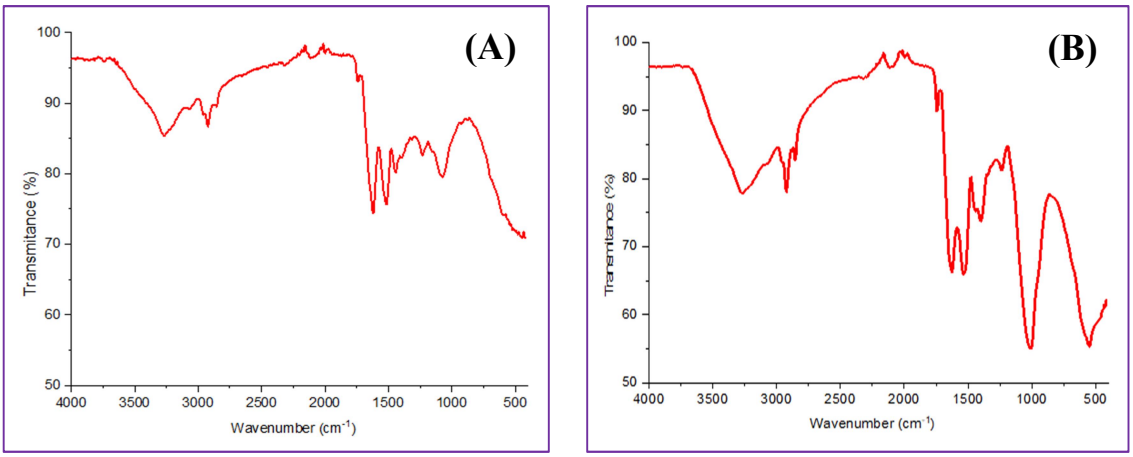


Figure 11 : FT-IR spectra of CP-ZnNPs **(A)** and WPZnNPs **(B)**

(Original photo)

1.1.3. SEM and EDX studies

The scanning electron microscopy (SEM) technique was utilized to visualize the size and shape of Zinc oxide nanoparticles. In Figure 12 The SEM (Phenom Prox) used SEM grids which were prepared by placing a small amount of sample powder on a copper coated grid and drying under lamp. In the formation of ZnONPs as well as their morphological dimensions in the SEM study the shape was oval and homogeneous with interparticle distance. Moreover, the shapes of the zinc nanoparticles proved to be multifaceted.

Energy dispersive X-ray (EDX) (Figure 13), showed that the biosynthesized ZnONPs powder by casein milk protein and whey milk protein had a size relatively less than 220 and 390 nm respectively. In the other side, EDX results show the presence of the elements oxygen, zinc, carbon, sulfur and nitrogen, which indicates the presence of components of the casein or whey protein bound to the elements of zinc (Table 5).

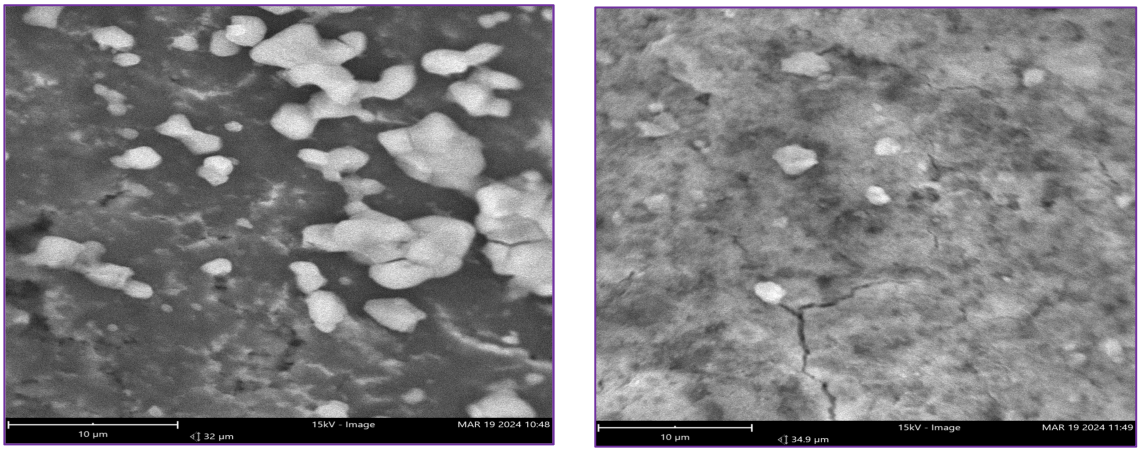


Figure 12: SEM micrographs of ZnO nanoparticles synthesized by casein milk protein **(On left)** and whey milk protein **(On right)**. **(Original photo)**

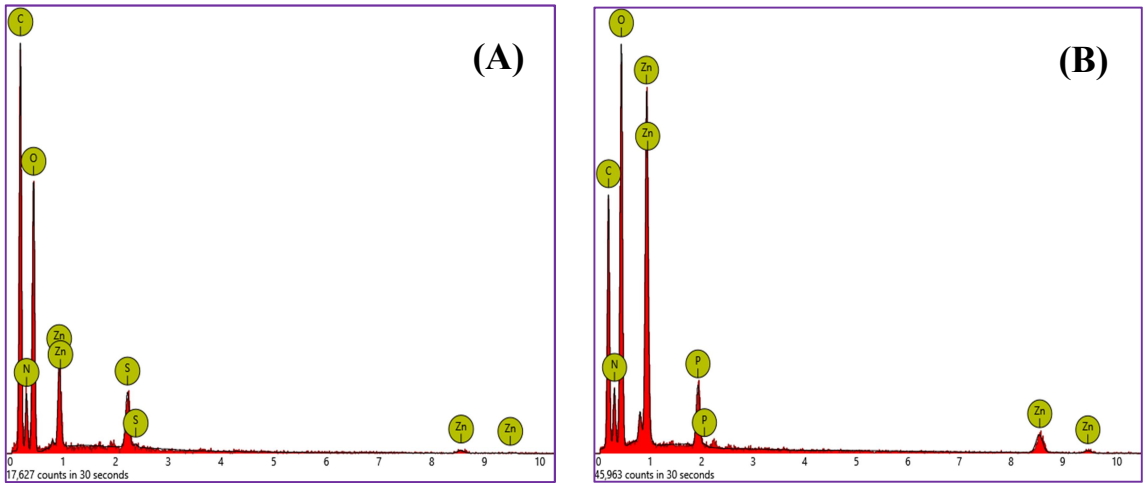


Figure 13: EDX analyses of the biosynthesized CP-ZnNPs **(A)** and WP-ZnNPs **(B)**

Table 5: The elemental composition analyses of the ZnNPs from the EDX plot of the SEM images CP-ZnNPs **(A)** and WP-ZnNPs **(B)**

(A) Element Number	**Element Symbol**	**Element Name**	**Atomic Conc.**	**Weight Conc.**
8	O	Oxygen	33.67	37.74
6	C	Carbon	44.77	37.67
7	N	Nitrogen	19.94	19.57
30	Zn	Zinc	0.60	2.73
16	S	Sulfur	1.02	2.29
(B) Element Number	**Element Symbol**	**Element Name**	**Atomic Conc.**	**Weight Conc.**
8	O	Oxygen	38.58	37.86
6	C	Carbon	40.01	29.47
30	Zn	Zinc	4.14	16.59
7	N	Nitrogen	16.07	13.80
15	P	Phosphorus	1.20	2.28

1.2. Protein determination

Based on Figure 14, the immobilization yield (IY) demonstrates a higher level in CP-ZnNPs (80.68%) compared to WP-ZnNPs (63.71%).

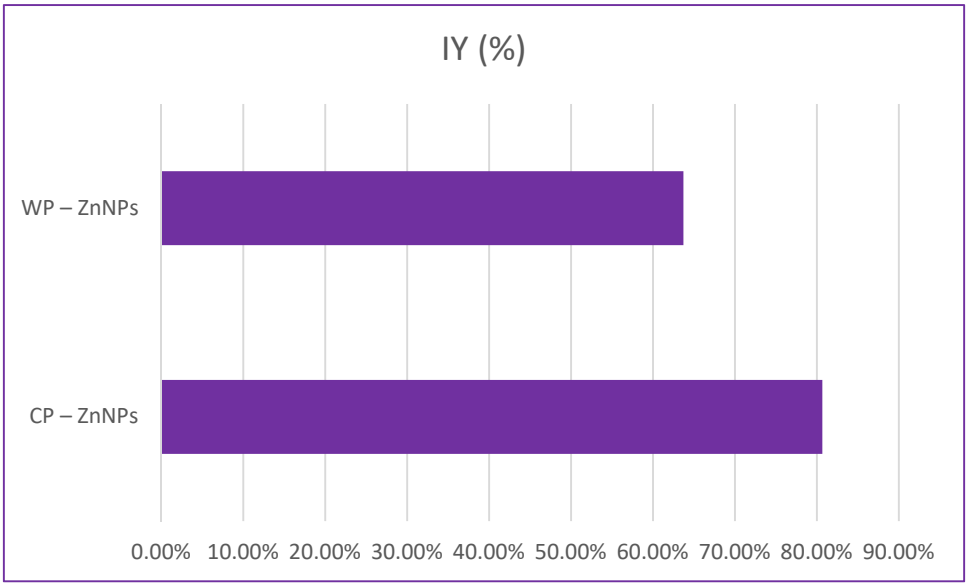


Figure 14: The immobilization yield of CP- ZnNPs and PL- ZnNPs.

1.3. Anti-oxidant activity (DPPH assay)

As demonstrated in Figure 15, CP-ZnNPs had a higher antioxidant activity compared to WP-ZnNPs.

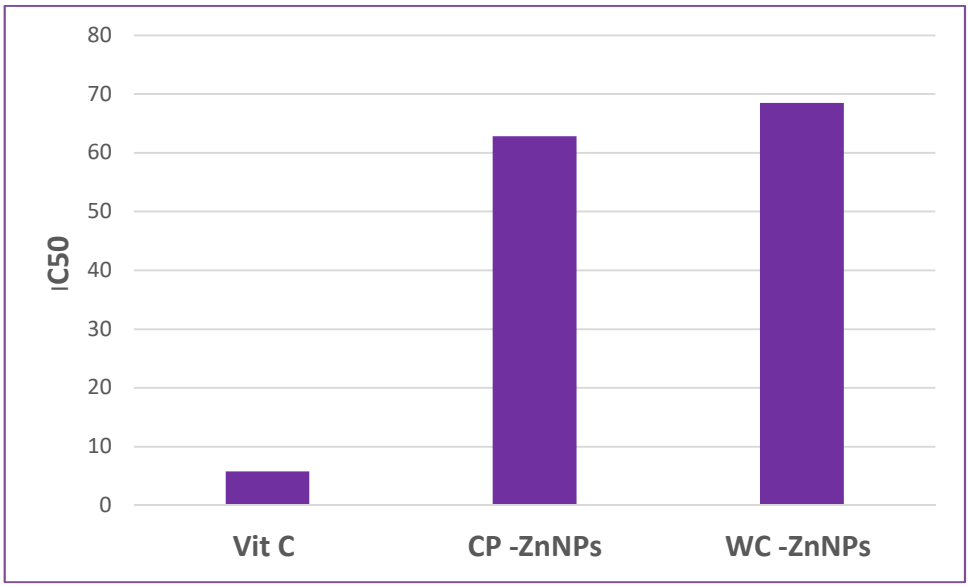


Figure 15: IC50 levels of antioxidant activity of Vit C, CP – ZnNPs and WP– ZnNPs.

1.4. Anti-inflammatory activity (Hemolysis assay)

As shown in Figure 16, WP-ZnNPs illustrated a higher anti-inflammatory activity in comparison to CP-ZnNPs .

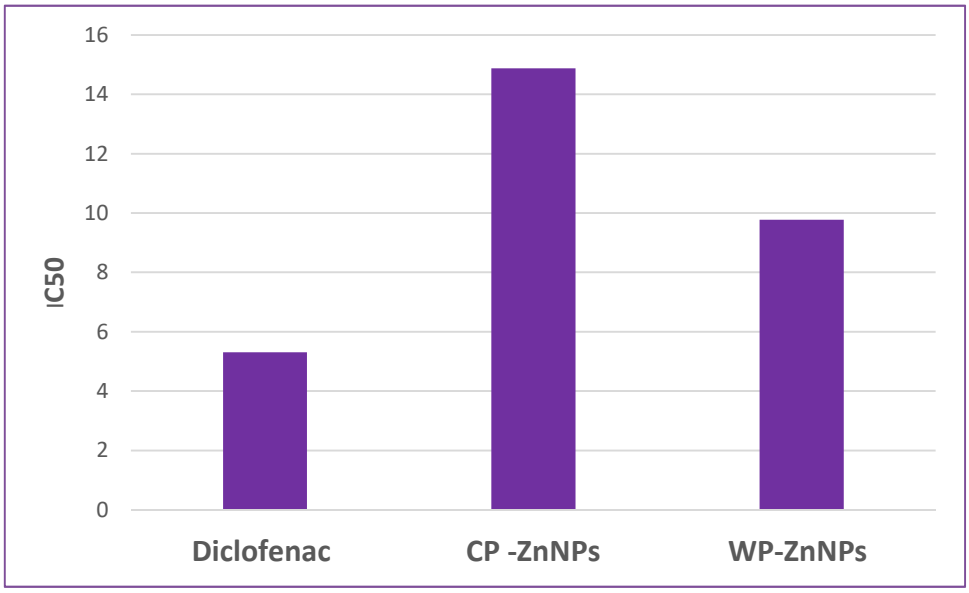


Figure 16: IC50 levels of anti-inflammatory activities of diclofenac, CP-ZnNPs and WP-ZnNPs.

2. *In-Vivo* study :

2.1. Growth parameters

After a period of treatment , results have shown that there is a significant loss of weight within CP-ZnNPs group ($p<0.001$) compared with control group and PHZ group. Also, the WP-ZnNPs group has shown a significant loss of weight ($p<0.05$) compared to the control group .Concerning the relative organs weight , compared to control group, it presents a significant increase for all organs but Liver in PHZ group , a significant decrease for all organs in CP-ZnNPs group and a development in only Kidneys relative weight for WP-ZnNPs group ($p<0.01$). Also, in comparison of PHZ group, CP-ZnNPs group's relative organs weight showed a significant loss for all of them but Testicles. As for WP-ZnNPs group , it demonstrated a significant gain of weight for all organs with the exception of Testicles ; a significant loss ($p<0.001$). More details are shown in (Table 6).

Table 6: Growth parameters for control and experimental groups

Parameters	Control (n = 5)	PHZ (n = 5)	CP-ZnNPs (n = 5)	WP-ZnNPs (n = 5)
Initial weight (g)	174.60 ± 5.62	167.40 ± 7.93	174.60 ± 7.66	174.20 ± 7.49
Weight Gain (g/day/rats)	1.1830 ± 0.0062	1.055± 0.165^{NS}	0.4550 ± 0.0791^{***c}	0.733± 0.183^{*NS}
Relative liver weight (%)	2.723± 0.0353	2.787± 0.0953^{NS}	2.479± 0.210^{*b}	2.672± 0.0478^{NSb}
Relative Kidneys weight (%)	0.28 ± 0.0108	0.375 ± 0.0380***	0.255 ± 0.0113^{**c}	0.308 ± 0.0250^{**c}
Relative Spleen weight (%)	0.238 ± 0.0105	0.382 ± 0.0568*	0.216 ±0.00965^{*c}	0.222 ±0.0139^{NSc}
Relative Heart weight (%)	0.306 ± 0.000667	0.35 ± 0.0210**	0.275 ± 0.0112^{**c}	0.301 ±0.0109^{NSc}
Relative Testicles weight (%)	0.558 ± 0.0909	0.777 ± 0.0914**	0.726 ± 0.152^{**NS}	0.535 ± 0.103^{NSc}

Values are mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001: significantly different from **control group**. a p<0.05, b p<0.01, c p<0.001 : significantly different from **PHZ group**. **NS :** No significance

2.2. Hematological parameters

- **Erythrocyte line in control and experimental groups :**

After the hematological analysis, precisely for the Erythrocyte line, results have shown in comparison to control group : a significant rise in all hematological parameters with a reverse results only for RBCs and MCHC in PHZ group . As for CP -ZnNPs , it illustrated a significant increase (p<0.001) for HGB , MCV , MCH and HCT (p<0.01) and a significant loss in RBCs (p<0.001). In addition, WP-ZnNPs group's results showed a significant increase (p<0.001) for all parameters with a significant decrease in RBCs and MCHC (p<0.001).Results compared to PHZ group have illustrated the following data: a significant rise (p<0.001) only for HGB and MCHC with a significant decline (p<0.001) for PLT in CP -ZnNPs. As for WP-ZnNPs, it demonstrated a significant development (p<0.001) for HBG , HCT and MCHC along with a significant loss for MCV, MCH and PLT as shown in (Table 7).

Table 7 : Erythrocyte line parameters' result in control and experimental groups

Parameters	Control (n = 5)	PHZ (n = 5)	CP -ZnNPs (n = 5)	WP-ZnNPs (n = 5)
RBC(10^6/µl)	9.383± 0.215	8.492 ± 0.221***	8.463± 0.0578*** NS	8.595 ± 0.223*** NS
HGB(g /dl)	15.2± 0.115	15.333± 0.0882**	16.4 ± 0.248***c	16.55 ± 0.05***c
HCT(%)	47.566±0.0882	49.266 ± 0.437***	51.025± 1.24**NS	52.275± 0.170***c
MCV(fL)	50.166±0.865	59.7 ± 0.757***	61.133± 1.02***NS	58.55± 0.750***b
MCH(pg)	16.65 ± 0. 141	19 ± 0. 138***	19 ± 0.063*** NS	18.5 ± 0.042*** c
MCHC(g/dl)	31.92 ± 0.139	31.22 ± 0.375*	32.433 ± 0.633NSc	31.65 ± 0.0957***c
PLT(10^3/µl)	702 ± 31.7	901.666 ± 6.65***	680.333± 15.6NSc	695.75± 24.9NSc

Values are mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001: significantly different from **control group**. a p<0.05, b p<0.01, c p<0.001 : significantly different from **PHZ group**. **NS :** No significance

- **Leucocyte line in control and experimental groups :**

For the Leucocyte line , results have shown the following data in comparison to the control group : a significant augmentation in WBC, Neutrophiles , Lymphocytes and P.Basophils levels for PHZ group .As for CP-ZnNPs, it demonstrated a significant increase in WBCs, Lymphocytes and P.Basophils. In addition, a significant decrease in WBCs, Lymphocytes and P.Eosinophils for WP-ZnNPs . In contrast with PHZ group, data illustrated that there was a significant loss (p<0.001) in Monocytes and P.Basophils for CP-ZnNPs group and a significant decrease in all parameters for WP-ZnNPs More detailed results are summarized in (Table 8).

Table 8 : Leucocyte line parameters' results for control and experimental groups

Parameters	Control (n = 5)	PHZ (n = 5)	CP -ZnNPs (n = 5)	WP-ZnNPs (n = 5)
WBC(10^3/µl)	7.325 ± 0.675	9.4 ± 1.30*	9.366 ± 1.20^{**NS}	6.7 ± 0.400^{*a}
Neutrophiles(10^3/mm^3)	1.332± 0.167	1.87 ± 0.0306***	1.526 ±0.348^{NSNS}	1.466 ± 0.264^{NSb}
Lymphocytes(10^3 /mm^3)	4.962± 0.550	6.19 ± 1.02***	5.5 ± 0.43^{***NS}	4.463± 0.192^{***c}
Monocytes(10^3 /mm^3)	0.07 ± 0.0122	0.408 ± 0.348^{NS}	0.09 ± 0.0321^{NSc}	0.065 ± 0.0144^{NSc}
P.Eosinophils(10^3 /mm^3)	0.136±0.0318	0.14 ± 0.0351^{NS}	0.11 ± 0.05^{NSNS}	0.063 ±0.0233^{***c}
P.Basophils(10^3 /mm^3)	0.73 ± 0.0531	1.46 ± 0.291***	0.995 ± 0.215^{**c}	0.742± 0.128^{NSc}

Values are mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001: significantly different from **control group**. a p<0.05, b p<0.01, c p<0.001 : significantly different from **PHZ group**. **NS :** No significance

2.3. Biochemical parameters

Biochemical markers have demonstrated divers results contrasted to control group; a significant rise (p<0.001) for all biochemical markers but TG and Iron which showed a significant decline (p<0.001) in PHZ group . As for CP -ZnNPs it illustrated a significant loss in TG, Urea, Crea, Asat, Iron (p<0.001) and Gly (p<0.01) with a significant rise in the rest of parameters. WP-ZnNPs results showed a significant increase (p<0.001) in Gly and Crea levels with a reversed significant result with the rest of parameters but Alat. On the other hand , when it comes to comparing with PHZ group, data showed a significant decrease for all markers but TC with a significant increase (p<0.01) in TG for CP-ZnNPs. As for WP-ZnNPs group it detected a significant decline (p<0.001) in all biochemical markers (Table 9).

Table 9 : Biochemical markers level in control and experimental groups

Parameters	Control (n = 5)	PHZ (n = 5)	CP -ZnNPs (n = 5)	WP-ZnNPs (n = 5)
Gly (g/l)	1.2533 ± 0.0082	1.7275 ±0.0637***	0.93±0.0923***c	1.5450 ±0.0061***c
TC(g/l)	0.75±0.00870	0.905± 0.00783 ***	0.9±0.00348*** NS	0.725± 0.0113*c
TG(g/l)	0.68 ± 0.0279	0.495 ±0.0148***	0.52667±0.0099***b	0.4425 ±0.00204***c
Urea(g/l)	0.5850 ± 0.0148	0.6350± 0.0131**	0.505± 0.00435***c	0.515± 0.00261*** c
Crea (mg/l)	13.1± 0.174	22.7 ± 1.6***	11.467 ± 0.348***c	15.125± 0.413***c
LDH(UI/ml)	490.75±2.22	693.8 ± 20.6***	578.8 ±10.6***c	388.9 ± 0.1***c
ALAT(UI/l)	34±0.211	41.5± 0.738***	34.5 ±1.16NSc	33.75±0.997NSc
ASAT(UI/l)	121±1.74	140±0.17***	84.50± 7.05***c	102.50± 0.96 ***c
Iron (µg/dl)	33.75±1.98	24.8±0.609***	12.433± 0.086***c	20.6± 0.261***c

Values are mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001: significantly different from **control group**. a p<0.05, b p<0.01, c p<0.001 : significantly different from **PHZ group**. **NS :** No significance

2.4. Oxidative stress parameters

2.4.1. Malondialdehyde (MDA) marker

MDA levels in comparison to control group showed a significantly high result in all organs for PHZ group. The same observation can be applied (p<0.001) for CP-ZnNPs (Liver & Spleen) and a significant low levels for Heart and Testicles. As for WP-ZnNPs group , it illustrated a significant decrease in all organs but spleen. Results contrasted to PHZ group for the same parameter, demonstrated the following data: a significant loss (p<0.001) in all organs but Kidneys for CP-ZnNPs. The exact same outcomes can be applied for WP-ZnNPs group as it illustrated in (Figure 17).

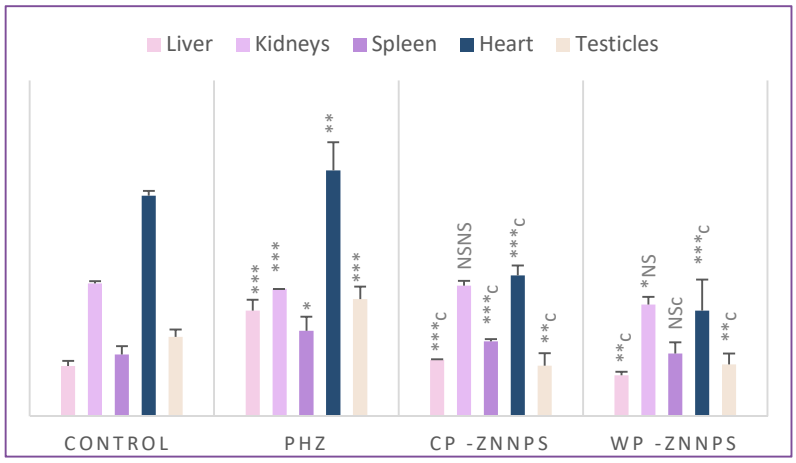


Figure 17: MDA levels in control and experimental groups

Values are mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001: significantly different from **control group**. **a** p<0.05, **b** p<0.01, **c** p<0.001 : significantly different from **PHZ group**. **NS :** No significance

2.4.2. Superoxide dismutase (SOD) marker

After comparing SOD levels to control groups, results shown that there was a significant increase (p<0.001) for all organs but the Heart which detected a significant loss (p<0.001) in PHZ group. Also, CP-ZnNPs demonstrated a significant rise only in Kidneys (p<0.01) and Spleen (p<0.001). Finally, Liver , Spleen and Testicles marked a significant augmentation a long with a significant decline (p<0.001) for Kidneys in WP-ZnNPs . On the other hand, with comparison to PHZ group , data showed a significant decrease in Kidneys (p<0.01) and Spleen (p<0.001) in CP-ZnNPs with a significant increase in the Heart (p<0.05). As for WP-ZnNPs there was a significant loss (p<0.001) for Kidneys and Spleen and an opposite outcome for Heart and Testicles (Figure 18).

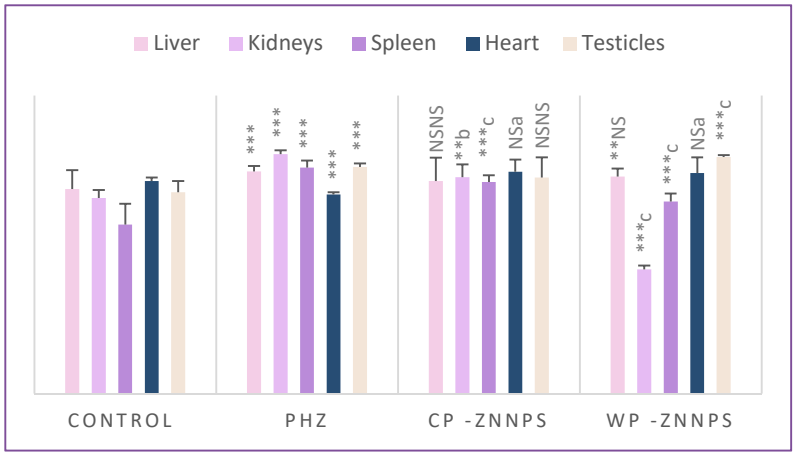


Figure 18: SOD levels in control and experimental groups

Values are mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001: significantly different from **control group**. **a** p<0.05, **b** p<0.01, **c** p<0.001 : significantly different from **PHZ group**. **NS :** No significance

2.4.3. Reduced glutathione (GSH) activity

Data demonstrated GSH levels in experimented groups in comparison to control group as following : a significant decrease in all organs but liver for PHZ group, a significant loss in liver ($p<0.001$) and Kidneys ($p<0.01$) for CP-ZnNPs and a significant rise ($p<0.01$) in Testicles along with opposite result in Kidneys ($p<0.01$) for WP– ZnNPs group. Nevertheless, results in comparison to PHZ group have shown that there was a significant development in all organs but Testicles for CP-ZnNPs. As for WP– ZnNPs group there was a significant rise ($p<0.001$) in all organs but Spleen (Figure 19).

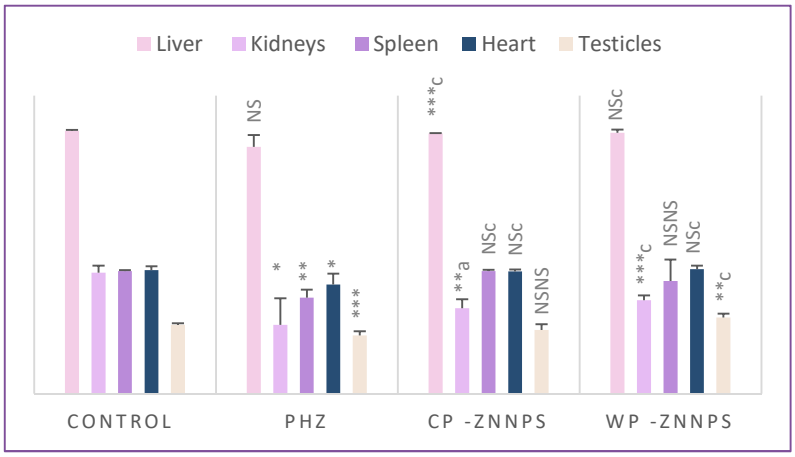


Figure 19: GSH levels in control and experimental groups

Values are mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$: significantly different from **control group**. **a** $p<0.05$, **b** $p<0.01$, **c** $p<0.001$: significantly different from **PHZ group**. **NS :** No significance

2.4.4. Catalase (CAT) activity

The last conducted test was CAT . Results of the experimental groups in contrast to control were analyzed as following : only Testicles have shown a significant increase in PHZ group . Kidneys and Testicles marked a significant loss along with a significant rise ($p<0.001$) in the Heart for CP-ZnNPs group . Liver also illustrated a significant decrease ($p<0.01$) on the contrary of Heart and Testicles ($p<0.001$) in WP-ZnNPs group . When it comes to comparing with PHZ group , data showed that there was a significant increase ($p<0.001$) in Spleen and Heart , however a significant loss ($p<0.05$) was marked for Testicles in CP-ZnNPs group . Lastly , results did show a significant decline for Liver and Testicles ($p<0.001$) and a reversed outcome for Spleen and Heart in WP-ZnNPs group (Figure 20).

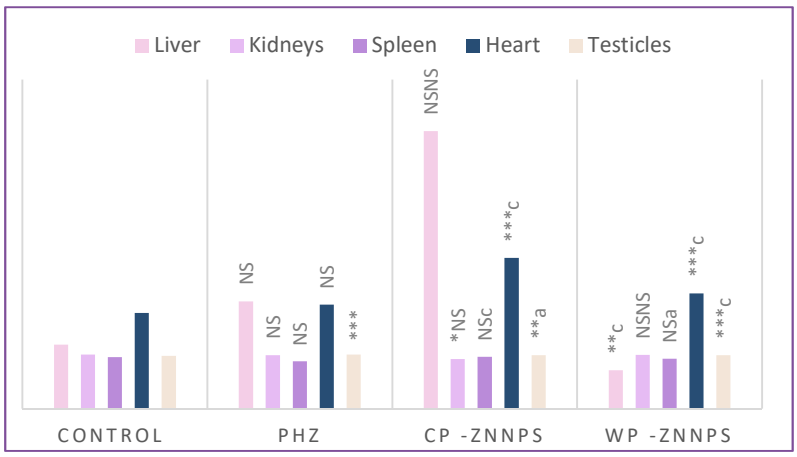


Figure 20: CAT levels in control and experimental groups

Values are mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001: significantly different from **control group**. **a** p<0.05, **b** p<0.01, **c** p<0.001 : significantly different from **PHZ group**. **NS :** No significance

2.5. Histopathological examination results

Figure 21, presents the rats' kidney photomicrographs from different groups. Light microscopic investigation of kidney section of control group reveal a normal renal cortex and glomerulus with tight Bowman's space, as well as proximal and distal convoluted tubules.

As a result of PHZ injections, the kidney exhibit several deleterious histological changes and lesions. The kidney section of the PHZ group show dilation of Bowman's space accompanied by hemorrhagic necrosis and infiltration of inflammatory cells surrounding the distorted glomerulus and tubules.

Moreover, the histological observations of the kidney section of the rats treated with CP-ZnNPs show completely restored and improved kidney architecture and renal glomerulus with normal structure. The tubules have a relatively regular, distinct lumen. The lobular organization of the glomerulus and a flat epithelium lining the glomerular capsule can be seen.

Finally, the histological results from the rats treated with WP-ZnNPs indicates a partial improvement in morphology, with the presence of necrosis and dilation of a Bowman's space due to the effect of PHZ.

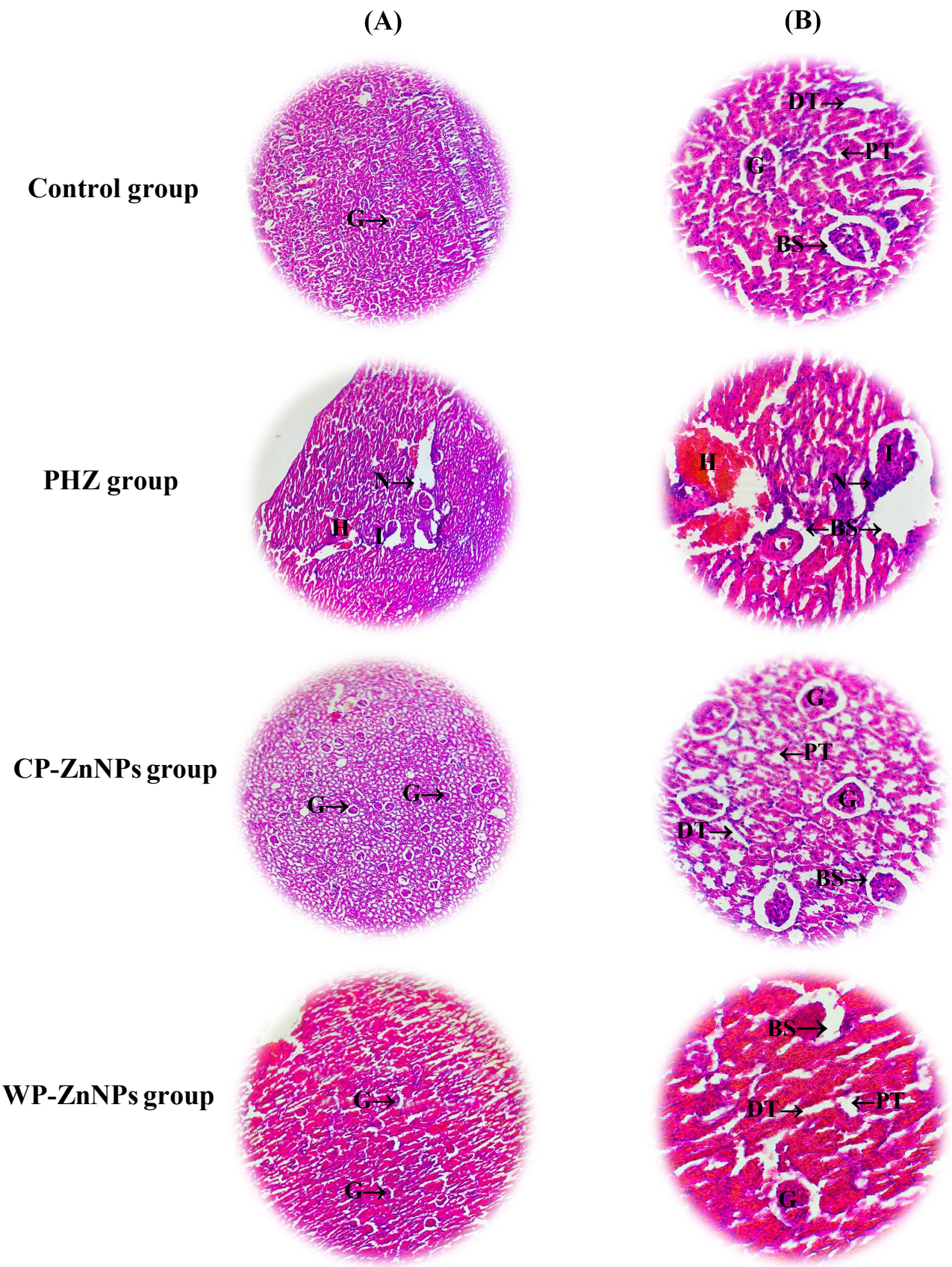


Figure 21: Representative photomicrographs of kidney sections of rats from the different experimental groups stained with hematoxylin and eosin stain (H&E), **(A)**: x10 and **(B)**: x40

G: Glomerulus, **BS**: Bawman's space, **PT**: Proximal tubules, **DT**: Distal tubules
I: Inflammation, **H**: Hemorrhage, **N**: Necrosis

Figure 22, illustrates the histopathology of the rats' spleen in the different groups. Light microscopic examination of the control group's spleen section reveals the normal architecture of the splenic pulp. The white pulp is composed of densely packed lymphocytes surrounding a central arteriole, while the red pulp consists of splenic cords interspersed with blood sinusoids in the marginal zone.

PHZ injections have resulted in numerous deleterious histological changes in the spleen. In the PHZ group, histological results indicate that a majority of the white pulp cells display degenerative changes characterized by vacuolated cytoplasm, numerous sub-capsular clear spaces, extensive necrosis accompanied by a high WBCs count , and some containing hemolyzed RBCs within the splenic parenchyma. Vacuolization was also observed in the wall of the central artery.

Further, spleen sections of rats treated with WP-ZnNPs show enhanced red and white pulps with a central arteriole, along with significant WBCs number and blood extravasation deposition.

Lastly, the spleen sections of rats treated with CP-ZnNPs indicate that the splenic tissues appeared nearly normal, displaying signs of recovery. Notable indications of complete vascular and tissue restoration were observed in the red pulp, white pulp, and marginal zone, compared with the previously examined groups where no hemorrhage or hemolysis was observed.

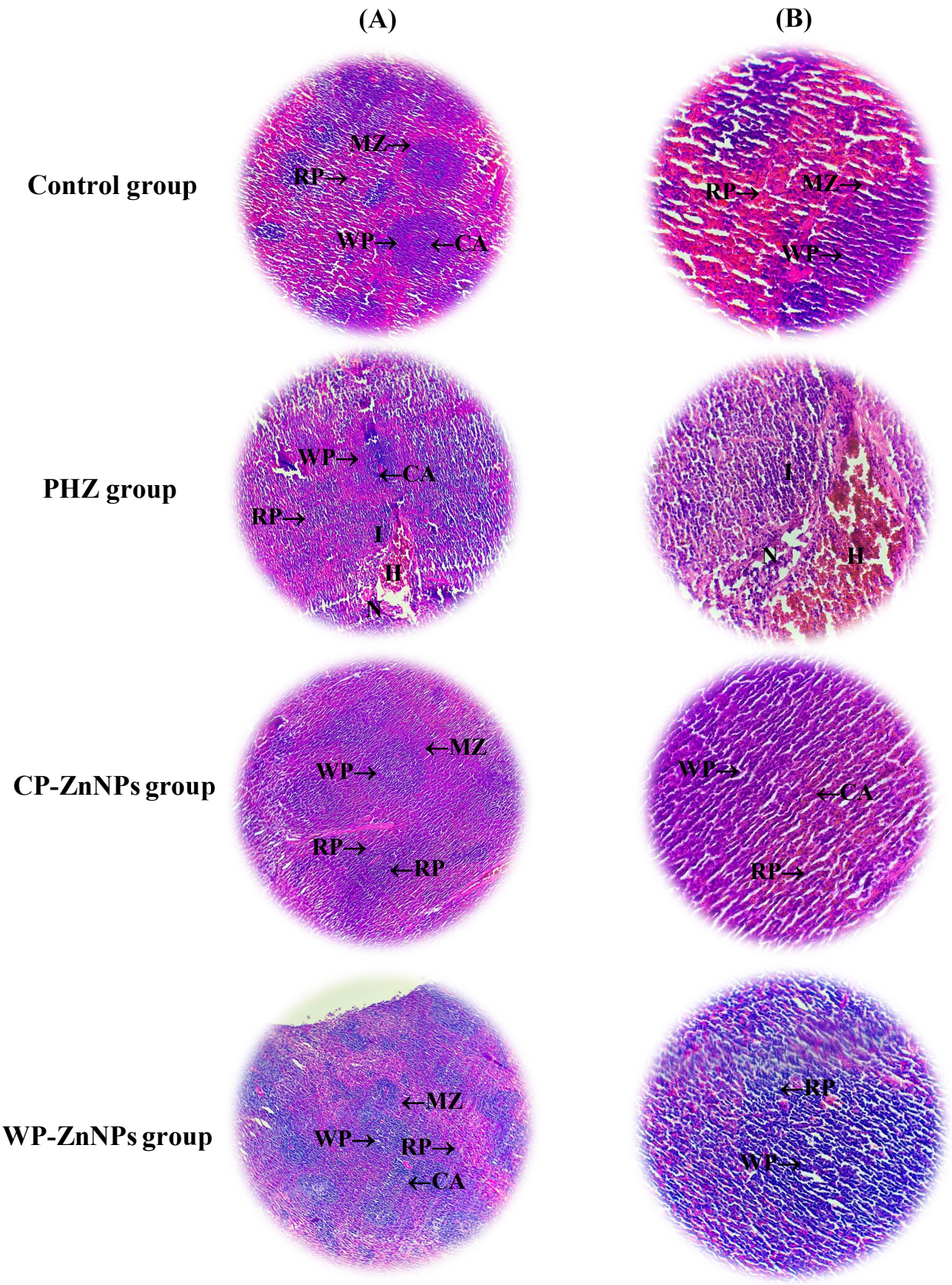


Figure 22: Representative photomicrographs of spleen sections of rats from the different experimental groups stained with hematoxylin and eosin stain (H&E), **(A)**: x10 and **(B)**: x40

RP: Red pulp, **WP**: White pulp, **CA**: central arteriole, **MZ**: Marginal zone
I: Inflammation, **H**: Hemorrhage, **N**: Necrosis

II. Discussion

A common public health issue known as anemia is defined as a decrease in hemoglobin concentration or erythrocyte mass in the blood, which results in a reduction in the blood's ability to carry oxygen (Manjegowda *et al.,* 2024). One drug with a toxic effect on red blood cells is phenylhydrazine (PHZ), which was used in inducing anemia in rats for this experiment. Due to PHZ's activation of reactive oxygen species production, it has been linked to oxidative stress which is involved in the aging and apoptosis of erythrocytes, thus inducing hemolysis (Ousaaid *et al.,* 2022). In order to heal this issues, we have used natural products formed of ZnNPs combined with milk proteins, precisely talking; casein and whey proteins. Several *in-vivo* and *in-vitro* tests were conducted to confirm the effects of these products against induced anemia by PHZ.

1. Characterization of Zinc oxide nanoparticles

The biosynthesized ZnONPs were characterized by different technics. UV-visible absorption spectroscopy is a powerful technique to examine the optical properties of nano-sized particles, also performed to further confirm the formation and stabilization of zinc oxide nanoparticles (Samy *et al.,* 2019). Our results indicate that ZnONPs absorb radiations at 300 nm. This result was very similar to another study indicate that the UV-visible spectrum showed a peak at 320 nm, which is specific for ZnONPs using *Cayratia pedata* leaf extract. Besides mentioned that for ZnONPs, the absorbance peak is reported between 310 nm and 360nm of wavelength (Jayachandran *et al.,* 2021). This also in the line with Fakhari *et al.,* (2019) stating the distinct peak was centered around 350 nm, which is specific for ZnNPs and due to their large excitation binding energy at room temperature.

FT-IR analysis was performed to identify the functional groups of the synthesized ZnO nanoparticles (Samy *et al.,* 2019). Results demonstrated the peak between 400-700 cm^{-1}. As represented in the study of Spoială *et al.,* (2021), the main adsorption peaks of the synthesized ZnNPs samples are observed at 462 cm^{-1} and 419 cm^{-1} and correspond to the stretching vibrations of the Zn-O bond.

For further characterization, the ZnONPs were analyzed by the Scanning electron microscopy (SEM) to observe their surface morphology and structure. The SEM image showed a spherical and multifaceted shape of zinc nanoparticles. A comparable result was reported by (Muhammad *et al.,* 2019). Our SEM analysis results also revealed a size relatively less than 220 and 390 nm in the biosynthesized ZnONPs powder by casein milk protein and whey milk protein, respectively. The large size of our ZnONPs is due to the combination of

zinc metal and animal protein (casein and whey milk proteins). As is known in biochemistry, proteins are macromolecules comprising linear polymers of amino acid residues joined by peptide bonds which exhibit different structures and compositions, giving different functional and nutritional properties (Ling & Hadinoto, 2022). On the contrary of (Chikkanna *et al.,* 2019) study while zinc nanoparticles synthesized using goat and sheep faecal matter (NPs GFM and SFM) exhibit lower particle size and possess nearly 40–120 nm for ZnONPs (GFM) and 60–130 nm for ZnONPs (SFM). Moreover, results of (Gur e *et al.,* 2022) study reported that detected Zn nanoparticle using *Thymbra spicata L.* plant sizes were determined to be between 6,5 nm and 7,5 nm.

Energy-dispersive X-ray spectroscopy (EDX) was used to find elemental composition and purity of ZnONPs samples. The EDX study showed the elements zinc and oxygen. The Zn content was 30 while O content was 8 of elements number, as proved by Shamim *et al.,* (2019) results. However, the results also indicated that ZnONPs weren't pure, there was traces of impurities such as carbon, nitrogen, phosphorus and sulfur. The presence of these elements is mainly due to the nanocomposite structure of ZnONPs contain organic materials which are casein and whey milk proteins used in ZnONPs biosynthesis. Furthermore, proteins represent one of milk's macroconstituents (along with water, lipids, and carbohydrates), accounting for ~ 3.0%–3.5% of the total composition (Goulding *et al.,* 2020). In addition, milk proteins are a collection of different mixtures which vary in structure and characteristics. They are classified into fractions of whey protein and clusters of casein. Casein has been the most significant protein in camel milk, with a fairly small ratio of whey proteins (Sumaira *et al.,* 2020).

Concerning determination of protiens, the Immobilization yield (IY) demonstrates a higher level in CP-ZnNPs (80.68%) compared to WP-ZnNPs (63.71%) which confirm the efficiency of the protein immobilization process in the zinc nanoparticles

2. Antioxidant activity (DPPH assay)

Result of the anti-oxidant activity studies have demonstrated that CP-ZnNPs exhibits a potent antioxidant effect compared to WP-ZnNPs. Our results indicated that ZnO nanoparticles with casein milk protein that were synthesized using green nanosynthesis could effectively scavenge the free DPPH radicals, thereby confirming their superior antioxidant activity.

We can explain this by the exogenous antioxidant property of camel milk proteins immobilized in the ZnNPs, as what was appeared in several studies by reducing oxidative

stress. It is proposed that the higher antioxidant activity of CM is likely to be due to the 6.7 times more vitamin C in fresh CM than fresh cow milk, and in addition to the presence of other antioxidant components such as caseins, Lactic acid bacteria (LABs), bioactive peptides, whey proteins, and especially lactoferrin. The antioxidant and the angiotensin-converting enzyme (ACE) inhibitor activity of camel total casein and camel b-caseins, increased after enzymatic hydrolysis. In fact, the produced peptides start to act as natural antioxidants and ACE-inhibitors when CM is consumed and digested.

Moreover, the antioxidant activity of casein hydrolysates of CM has significant inhibitory activity in 2,2-diphenyl-1- picryl-hydrazyl-hydrate (DPPH) assay. Hydrolysis conditions and enzymes used affect the DPPH radical scavenging activity of the whey protein hydrolysate (Behrouz *et al.,* 2022).

This result is also corroborated previous findings, that reported the antioxidant potential was measured using DPPH free radical scavenging assay in the *B. juncea* leaves under the different ZnONPs treatments. Amendment of *B. juncea* plants using ZnONPs revealed that antioxidant activity in leaves was significantly boosted from 54% (in 20 mg L-1 of ZnONPs) to 97% (in 200 mg L-1 of ZnONPs) relative to the control (35%). Overall, ZnONPs have a positive impact on antioxidant activities of different plant species. The antioxidant activity in the mustard leaves of plants provided with different concentrations of biosynthesized ZnONPs confirms that the addition of ZnONPs promotes the biosynthesis of compounds with antioxidant activity like flavonoids and phenols (Singh *et al.,* 2018; Iziy *et al.,* 2019; Geremew *et al.,* 2023).

3. Anti-inflammatory activity (Hemolysis assay)

Based on the anti-inflammatory assay results, WP-ZnNPs illustrated a higher anti-inflammatory activity in comparison to CP-ZnNPs and to the diclofenac sodium as a positive control. This result is in agreement with the study of El-Fakharany *et al.,* (2023) stating that lactoferrin-coated zinc nanoparticles (Lf-Zn-NPs) revealed therapeutic efficiency such as anti-inflammatory activity. Moreover, another research reported that ZnONPs have an interesting anti-inflammatory property in response to pathogens (Carrouel *et al.,* 2020).

Besides, these findings can be explained by the ability of CM proteins in reducing inflammation. In general, the anti-inflammatory effects of unfermented and fermented CM are mainly due to the high content of antioxidant components such as lactoferin, vitamin C, bioactive peptides, and whey proteins (Izadi *et al.,* 2019). Likewise, CM has anti-inflammatory effects against infectious diseases (Mohammadabadi, 2020).

4. Growth parameters

In addition to causing anemia, PHZ can also induce hypertrophy in several organs, primarily due to its effects on oxidative stress and iron metabolism. Our results have demonstrated a significant increase in relative organs weights in PHZ group compared to control group. It is known that Spleen is heavily involved in the recycling of RBCs. The increased hemolysis caused by PHZ leads to an increased workload for the spleen, resulting in spleenic hypertrophy. The last organ becomes enlarged due to the increased need to filter damaged RBCs and manage the resulting iron overload (Gensluckner *et al.,* 2024). Moreover, the liver is a primary site for detoxification and iron storage. PHZ-induced oxidative stress and iron overload can cause liver hypertrophy as the organ attempts to manage the increased iron load and repair oxidative damage (Gensluckner *et al* ,. 2024). While less commonly discussed, the kidneys can also undergo hypertrophy as they are involved in filtering the blood and managing the increased oxidative stress and metabolic byproducts resulting from hemolysis and iron overload (Wang *et al.,* 2024). The heart, being highly vascularized, is particularly susceptible to damage from reactive oxygen species (ROS) and inflammatory cytokines. This stress can lead to hypertrophic responses as the heart attempts to adapt to increased metabolic demands and oxidative damage (Derouiche *et al.,* 2022). Testicular tissue is also vulnerable to oxidative damage. ROS can impair spermatogenesis and steroidogenesis, leading to compensatory hypertrophy as the tissue attempts to maintain normal function despite ongoing cellular damage (Wang *et al.,* 2024). Overall, PHZ-induced organ hypertrophy is primarily driven by oxidative stress, iron overload, and subsequent inflammatory responses. These factors lead to cellular and tissue-level adaptations as organs strive to manage the increased metabolic and oxidative burden. The results also showed a significant decrease in organs relative weight for CP-ZnNPs and WP-ZnNPs groups compared to PHZ group and a similarity for control group. This can be exWPained by knowing that Zinc is crucial for maintaining cellular integrity and function (Chetehouna *et al.,* 2020). ZnNPs contribute to the stabilization of cell membranes and the prevention of cellular apoptosis, particularly under stress conditions as confirmed in the previous study. This protective role is vital in preventing pathological changes in cells that can lead to hypertrophy. By maintaining cellular homeostasis, ZnNPs help in preserving normal tissue structure and function (Liu *et al.,* 2023). Additional, Milk proteins, particularly whey and casein, have been studied for their potential roles in preventing hypertrophy in various organs because of containing bioactive peptides that have anti-inflammatory effects. Chronic inflammation is a key factor in the development of hypertrophy in organs. By reducing inflammation, milk

proteins can help prevent or mitigate hypertrophy in these organs (McGregor *et al.,* 2013). As they are rich In amino acids like cysteine, which is a precursor to glutathione, a powerful antioxidant. Antioxidants help to neutralize reactive oxygen species (ROS) and reduce oxidative stress, which is linked to hypertrophy and organ damage (Møller & Bernardi, 2013) as it was clearly observed in histological assays for the current study .

5. Hematological parameters

After conducting the hematological analysis, particularly focusing on the erythrocyte line, the results have been compared to those of the control group showed that there was a significant rise in all Hematological parameters with a reverse results only for RBC and MCH in PHZ group. On the other hand Leucocyte line results have also shown a significant augmentation in WBCs, Neutrophiles , Lymphocytes and P.Basophils levels for PHZ group. These data can be explained after knowing that PHZ treatment leads to a significant reduction in the erythrocyte count. This is due to its ability to cause oxidative stress and damage to the red blood cell membrane, resulting in hemolysis as explained in (Camaschella, 2019). Knowing that red blood cells are destroyed, hemoglobin levels drop. Hemoglobin is the protein in red blood cells responsible for oxygen transport, and its reduction indicates the severity of anemia induced by PHZ (Camaschella, 2019). Additionally, hematocrit levels, which measure the proportion of blood volume occupied by red blood cells, are significantly reduced. This reflects the overall decline in red blood cell mass (Rabindrakumar *et al,.* 2018). Furthermore, MCV may increase due to the larger size of reticulocytes compared to mature red blood cells. Elevated MCV suggests the presence of regenerative anemia, where the body attempts to produce new red blood cells rapidly (Rabindrakumar *et al.,* 2018) as it is detected in our case. Since PHZ-induced hemolysis leads to increased oxidative stress and inflammation (Wang *et al.,* 2024), WBCs increased level is an expected outcome which indicates an immune response to the damage inflicted on red blood cells (Rabindrakumar *et al.,* 2018).

Treatment groups towards this issues have demonstrated a significant rise ($p<0.001$) only for HGB and MCHC with a significant decline ($p<0.001$) for PLT in CP-ZnNPs. As for WP-ZnNPs, it demonstrated a significant development ($p<0.001$) for HBG, HCT and MCHC along with a significant loss for MCV, MCH and PLT in addition to a significant loss ($p<0.001$) in Monocytes and P.Basophils for CP-ZnNPs group and a significant decrease in all parameters for WP-ZnNPs. In order to explain these outcomes, a research has confirmed that Zinc nanoparticles can modulate the immune response by influencing the production of

cytokines, which are proteins involved in inflammation. They help in reducing the levels of pro-inflammatory cytokines such as TNF-α, IL-6 and increase anti-inflammatory cytokines like IL-10 along with its ability of supporting the production and maturation of red blood cells (Runthala *et al.,* 2023). Moreover, These milk proteins can modulate immune responses by enhancing the activity of natural killer cells and macrophages, thus reducing inflammation (Ning & Zeller,2019) which can lead to an amelioration in anemia's level in the targeted subject .

6. Biochemical Markers

After PHZ injecting, the studied biochemical parameters demonstrated a significant rise ($p<0.001$) for all parameters with the exception of TG and Iron level which showed a significant decline ($p<0.001$) in PHZ group. On the other hand, and after the injecting of treatment in the experimental groups, results showed a significant decline ($p<0.001$) in all biochemical markers for WP-ZnNPs group, as it is also the case for CP-ZnNPs group with the exception of a significant increase ($p<0.01$) in TG in comparison of PHZ group. Similar results were found in (da Conceição *et al.,* 2022) and (Ezugwu *et al.,* 2022). Hepatic cells contain higher concentrations of AST and ALT in the cytoplasm and AST, in particular ,exists in the mitochondria. Damage or assault to hepatic cells induces leakage of plasma leading to an increased level of hepato-specific enzymes in serum. The measurement of serum Asat and Alat levels serve as means for indirect assessment of liver function. Urea and creatinine are non-electrolytes found in the body (Ezugwu *et al.,* 2022). Urea is produced from metabolism of amino acid and creatinine is formed from the metabolism of muscle creatinine and creatine phosphate. They are both excreted by the kidneys. The excretion of urea and creatinine is used to ascertain renal function (Ezugwu *et al.,* 2022). This process is actively carried out by the kidneys. Creatinine is usually produced at a fairly constant rate by the body and filtered out of the blood by the kidneys. If the filtering capacity of the kidney is deficient, creatinine blood levels rise. In general, increased urea levels are associated with nephritis, renal ischemia and urinary tract obstruction (Ezugwu *et al.,* 2022). Urea is the major end product of protein catabolism in mammals and is the primary vehicle for removal of toxic ammonia from the body. It is primarily produced in the liver and secreted by the kidneys (Oluwole *et al.,* 2012). From this present work, the damage caused by PHZ administration was reversed in groups treated with CP-ZnNPs and WP-ZnNPs. According to (Dikhanbayeva *et al.,* 2021), these treatments can repair and adequately protect liver tissue through membrane-stabilizing and leakage prevention of intracellular enzymes. Moreover, as clearly known that the distribution of nanominerals in the body is greater than inorganic and organic minerals due to its high

bioavailability that allows nanominerals to pass through the small intestine (Ramiah *et al.,* 2019). Along with Zn's work as cofactors for antioxidant enzymes and the fact that Zn binding protein, in our case camel milk casein and whey proteins ,is an effective scavenger of hydroxyl radicals that protect against immune-mediated free radical attack as approved by the previous authors (Derouiche *et al.,* 2017) which can explain the finding results.

7. Oxidative stress

Oxidative stress occurs when the level of harmful reactive oxygen species (ROS) surpasses the body's antioxidant defense. Antioxidants have the capacity to scavenge free radicals and harmful oxygen-derived species such as hydroxyl radicals, singlet oxygen, and hydrogen peroxide. This ability enables them to prevent or alleviate damage to cells and the negative impacts of diseases caused by oxidative stress (Bruno *et al.,* 2023). The present evidence suggests that CP-ZnNPs and WP-ZnNPs enhanced the activity of antioxidant enzymes which possibly protected the *Wistar* rats from induced oxidative stress resulted from anemia.

As it is the case in our PHZ group with a significant rise in MDA level in all previously mentioned organs. The elevated level of MDA detected in animals exposed to PHZ is an indication that PHZ induced tissue and cellular injury. The significant loss ($p<0.001$) of MDA levels in CP-ZnNPs and WP-ZnNPs groups is attributable to their antioxidant activity to mitigate cellular and tissue injury, as it is the case in (Bruno *et al.,* 2023).

Additionally, a significant increase ($p<0.001$) in SOD levels in PHZ group compared to control. Which can be explained by its ability of converting superoxide radical($O_2^{\cdot -}$) to hydrogen peroxide (H_2O_2) in order to reduce oxidative stress (Hanqing *et al.,* 2021). As for CP-ZnNPs group it illustrated a significant decrease in Kidneys ($p<0.01$) and Spleen ($p<0.001$) in similarity with WP-ZnNPs group as it shows in (da Conceição *et al.,* 2022).

Our experiment also tested the GSH levels which detected a significant development in all organs for CP-ZnNPs. Likewise in WP-ZnNPs group with a significant rise ($p<0.001$), as it is the case in (Kale *et al.,* 2019; Derouiche *et al.,* 2022). Higher levels of GSH indicate a robust antioxidant defense system. This can help neutralize reactive oxygen species (ROS) and prevent oxidative damage to cells and tissues (Townsend & Tew, 2019). Moreover, GSH is essential for the functioning of various enzymes and for maintaining the integrity of cell membranes. Increased GSH levels can support these functions, promoting overall cellular

health (Meister & Anderson 2019).

The last conducted marker was CAT with a significant rise ($p<0.001$) in both CP-ZnNPs and WP-ZnNPs groups compared to PHZ as it was also shown in (da Conceição *et al.,* 2022; Derouiche *et al.,* 2022). An increase in CAT activity often indicates an adaptive response to elevated levels of reactive oxygen species (ROS) in an attempt to mitigate oxidative stress by the detoxification of hydrogen peroxide (H_2O_2) by converting it into water and oxygen (Wang *et al.,* 2021). All the previous founding concluded that the treatment applied in the experimental groups did show a remarkable change concerning the biochemical parameters in improving the health of lab rats by decreasing the anemia levels .

8. Histological analysis

After inducing anemia by PHZ injection into the experimental groups it demonstrated the expansion of bowmen's space with hemorrhagic necrosis and inflammation at the level of kidney tissues, along with necrosis accompanied by a high number of WBCs at the level of Spleen tissues, which confirms the presence of inflammation, similar outcomes were found in (Kale *et al.,* 2019; Ezugwu *et al.,* 2022). The Spleen is a repository for dead RBCs, and it is also where Hb is broken down. Hemolytic anemia causes an increase in iron deposition in the Spleen due to the accelerated breakdown of hemoglobin demonstrated that the cause of necrosis in PHZ-treated groups (Soliman *et al.,* 2023).

The administration of the treatment significantly reversed these results; as data showed a partial correction in morphology in both kidneys and Spleen for WP-ZnNPs and a mainly total recovery for CP-ZnNPs. Similar results were observed within whey protein in this study (da Conceição *et al.,* 2022). Another study may explain these results saying that casein is used as a pro-inflammatory molecule induces chemotaxis of granulocytes and macrophages and induces the accumulation of myeloid progenitor cells in bone marrow (Ledesma-Martínez *et al.,* 2019). Bone marrow progenitor cells cultured with interleukin 3 (rmIL-3) as a growth factor in the presence of casein show increased cell numbers that exceed 50% as it shown in the same study. Moreover, It has been demonstrated that in hematopoietic cells such as polymorphonuclear cells and monocytes there are specific receptors for caseins (Ledesma-Martínez *et al.,* 2019). This article also illustrated that it has recently been shown that α-casein binds to toll-like receptors (TLRs) thus, casein could exert immunomodulatory effects on leukocytes and even participate in the genesis of blood cells via TLRs.

Conclusion

Conclusion

Anemia is a major public health issue. It's an extremely common disease affecting up to one-third of the global population. Anemia is a condition that happens when your body doesn't have enough healthy red blood cells or hemoglobin. The main component of blood is hemoglobin which carries oxygen to the cells. Its reduction causes decrease in the availability of oxygen in the body thereby causing anemia. Macrocytic anemia is a blood disorder that happens when your bone marrow produces abnormally large red blood cells. These abnormal blood cells lack nutrients red blood cells need to function normally. There are two types of macrocytic anemia. They develop when your body lacks certain nutrients. People may develop macrocytic anemia when they don't get enough vitamin B12 and/or folate (vitamin B9) to create healthy red blood cells, or they have medical conditions that prevent their bodies from absorbing those nutrients. The symptoms generally includes shortness of breath, dizziness, headache, chest pain, pale skin, increase in heartbeat.

However, based on the results obtained, we can conclude that:

- ✓ The biosynthesis of zinc oxide nanoparticles (ZnONPs) using casein (CP- ZnNPs) and whey (WP-ZnNPs) proteins from camel milk highlights their potential as natural nanoparticle stabilizers which have been characterized by various methods, including UV-VIS spectroscopy, FT-IR spectroscopy, and SEM/EDX analysis.

- ✓ The *in-vitro* study on CP-ZnNPs and WP-ZnNPs has shown significant antioxidant and anti-inflammatory properties, suggesting these nanoparticles could be valuable biological and medicinal agents for treating various diseases linked to oxidative stress and inflammation.

- ✓ CP-ZnNPs and WP-ZnNPs, have confirmed, once again, their potential roles in preventing hypertrophy in various organs because of containing bioactive peptides that have anti-inflammatory effect along with their contribution in stabilizing cell membranes and preventing cellular apoptosis.

- ✓ Hematological analysis showed that there was a significant change between the animatic rats and the ones treated with CP-ZnNPs and WP-ZnNPs by showing their effects on decreasing the anemia indexes levels and modulating the immune responds which lead to reducing inflammation.

- ✓ According to the biochemical analysis , the damage caused by PHZ administration in rats was reversed in groups treated with CP-ZnNPs and WP-ZnNPs which means that this treatment can repair and adequately protect the body organs.

- ✓ The present evidence concluded that CP-ZnNPs and WP-ZnNPs enhanced the activity of antioxidant enzymes which possibly protected the *Wistar* rats from induced oxidative stress resulted from induced Anemia.

- ✓ Finally, the effect of the studied treatments broadened into the microscopic level which demonstrated a partial healing in the studied tissues for WP-ZnNPs group and a total recovery for CP-ZnNPs .

Over all, this study can be a prove to confirm the effect of WP-ZnNPs and CP-ZnNPs on reducing Anemia after passing all needed tests which can lead to a path of natural and more effective alternatives cures to the mentioned health issue.

Perspectives

To deeply form a comprehensive study on how WP-ZnNPs and CP-ZnNPs reduce anemia, we can consider multiple perspectives :

- ✓ Evaluating the effectiveness of WP-ZnNPs and CP -ZnNPs in treating anemia in various demographic groups (e.g., children, pregnant women, elderly).

- ✓ Assessing the safety profile and potential toxicological effects of long-term use of these nanoparticle-protein complexes.

- ✓ Investigate the impact of these treatments on vitamins (e.g., Vitamin C, B12, B9) uptake.

- ✓ Comparing the effectiveness of WP-ZnNPs and CP-ZnNPs with traditional iron supplements and other novel therapies.

- ✓ Investigate the effect of WP-ZnNPs and CP-ZnNPs on various chronic or acute diseases.

- ✓ Testing the anti-cancer activity on the formed treatment.

- ✓ Creating an hybrid nanoparticles based on both casein and whey proteins together.

- ✓ Studying the effect of these formulations on other diseases.

- ✓ Perform health economic evaluations comparing the costs and benefits of these treatments to traditional anemia therapies.

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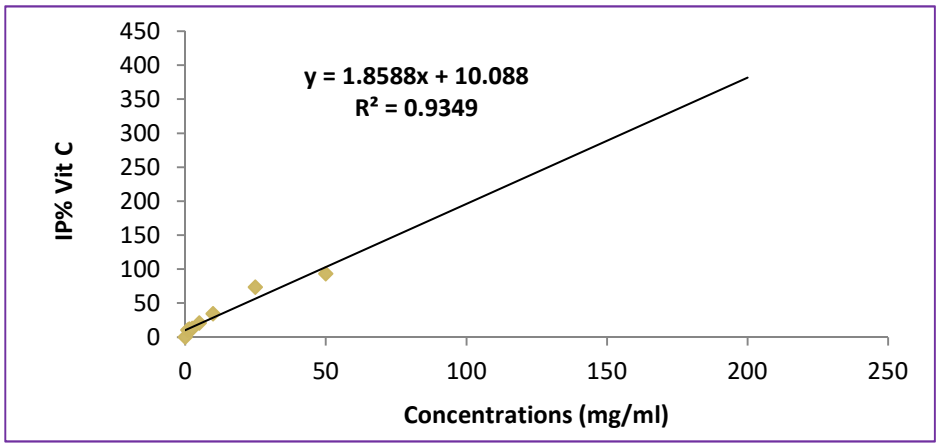
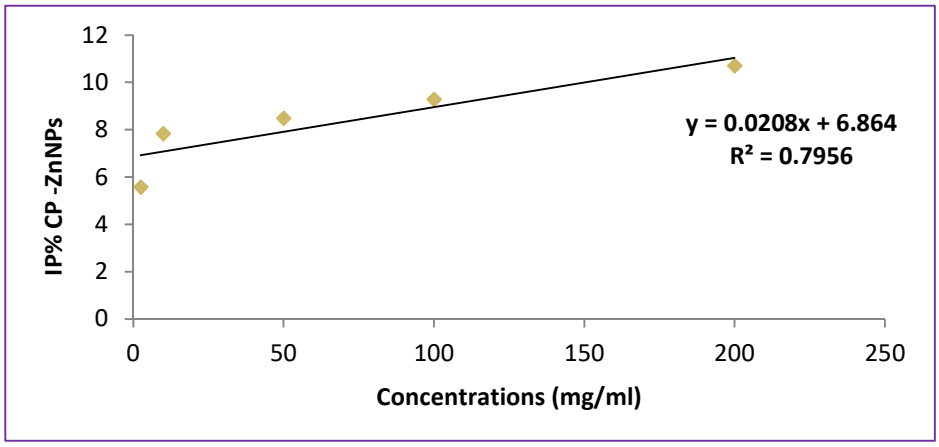
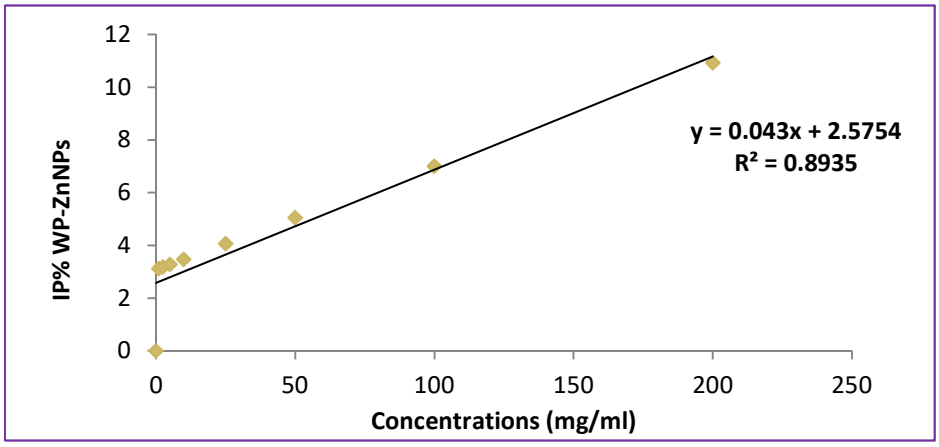
Annexes



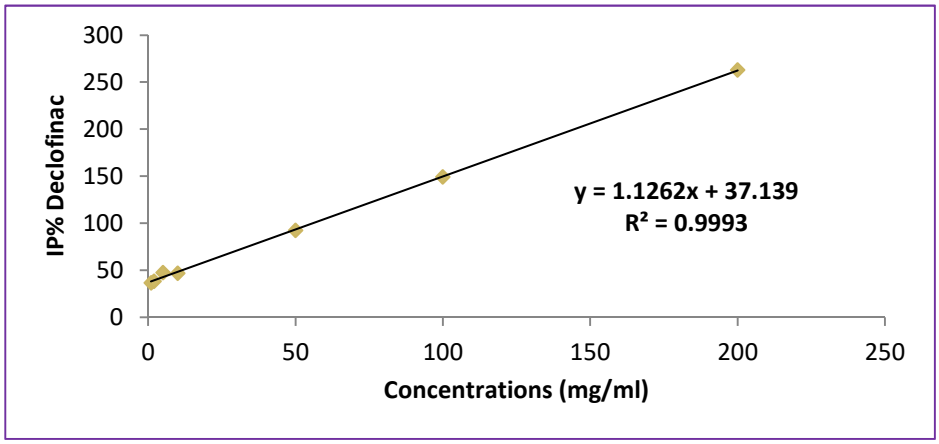
Annexe 1 : IP of DPPH test in different concentrations of Vit C.



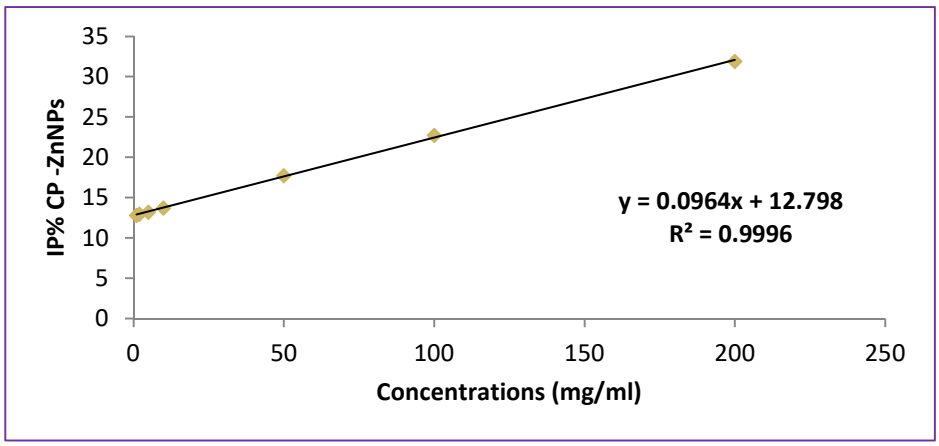
Annexe 2 : IP of DPPH test in different concentrations of CP –ZnNPs.



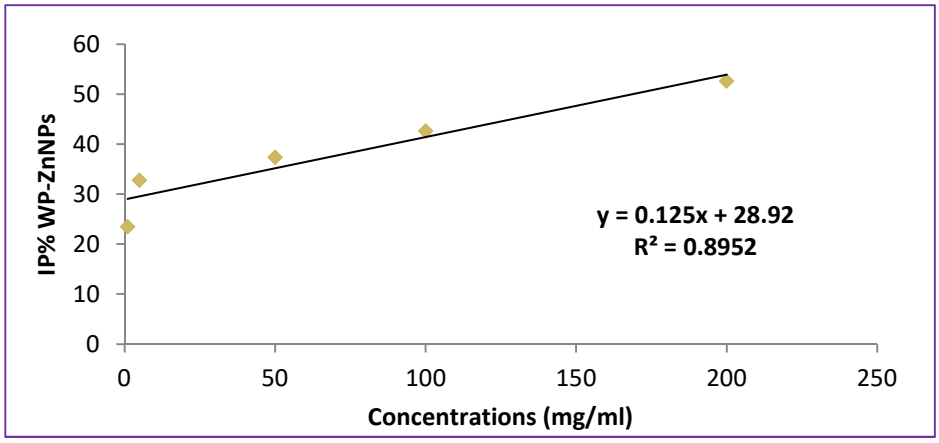
Annexe 3 : IP of DPPH test in different concentrations of WP-ZnNPs.



Annexe 4 : IP of Hemolysis assay in different concentrations of Declofinac.



Annexe 5 : IP of Hemolysis assay in different concentrations of CP-ZnNPs.



Annexe 6 : IP of Hemolysis assay in different concentrations of WP-ZnNPs.