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Phytosynthesis of CuNPs by *Medicago sativa* and their effects on some biological and oxidative stress parameters in experimental Anemia induced by phenylhedrazine in rats

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Dedicate

*We dedicate this work and our gratitude to our fathers (**Hossein, brahim, Ahmed, Abdel Hamid**) and our mothers (**Fatima, Najat, Fatiha, Yasmina**), to whom honor falls astray and unable to honor you, to those who learned diligence, perseverance, love of knowledge and walking in the footsteps of beloved Mustafa. And our husbands (**Ali, Mohammad**) we give them all our thanks and appreciation for supporting us in the throes of this work with patience and endurance.*

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Abstract

This investigation was aimed to study the effects of the aqueous extract of *Medicgo sativa* and copper nanoparticles CuNPs on certain biochemical, hematological and oxidative stress parameters in phenylhydrazine induced experimental anemia in female wistar rats. The experimental study carried out in the laboratory on 20 female Wistar rats were divided into four groups, the first group rats serves as a control, the second is experimental anemia rats (PHZ), and the third is experimental anemia rats treated by copper nanoparticles (3 mg/kg) (PHZ+CuNPs) and the fourth is experimental anemia rats treated with the aqueous extract of *M. sativa* (200 mg/kg) (PHZ+MS). Some biochemical, hematological and oxidative stress markers were determined. Biosynthesized CuNPs were characterized by analytical methods. Results of in vitro phytochemical and HPLC analysis results revealed that *M. sativa* contains most of active compounds, especially phenolic (naringin and vanilic acid) with high antioxidant activity. Characterization of CuNPs confirmed the involvement of biological molecules in CuNPs synthesis with size ranged from 19.8 to 92.8 nm. Results of in-vivo rats study showed a change in hematological and some biochemical parameters in experimental anemia group compared to control. Also, results illustrated an oxidative stress in PHZ rats with high level of MDA and low level of SOD compared to control. Histopathology of bone, spleen and kidneys tissues tested confirmed the altered in these tissues with inflammation, necrosis, hemorrhagic in anemia rats compared to control. However, the obtained Results show a partial improvement of all of previous alteration in rats treated with *M. sativa* plant and CuNPs compared to infected rats. In conclusion, the present study suggests that CuNPs and *M. sativa* extract could be a substantially promising anti-inflammatory and antioxidant effect which can be used in treatment of anemia.

Keywords: Anemia, Phenylhydrazine, *Medicago sativa*, CuNPs, , Oxidative stress, Rats.

Résumé

Cette étude visait à étudier les effets de l'extrait aqueux de *Medicgo sativa* et des nanoparticules de cuivre CuNPs sur certains paramètres biochimiques, hématologiques et de stress oxydatif dans l'anémie expérimentale induite par la phenylhydrazine chez des rattes Wistar. L'étude expérimentale menée au laboratoire sur 20 rattes femelles qui a été divisée en quatre groupes, le premier groupe rattes sert au témoin, le second est pour des rattes ont l'anémie expérimentale (PHZ), et le troisième est pour des rattes ont une anémie expérimentale traitée par des nanoparticules de cuivre (3 mg/kg) (PHZ+CuNPs) et le quatrième est pour des rattes ont l'anémie expérimentale traitée par l'extrait aqueux de *M. sativa* (200 mg/kg) (PHZ+MS). Certains marqueurs biochimiques, hématologiques et de stress oxydatifs ont été déterminés. Les CuNPs biosynthétiques ont été caractérisés par des méthodes analytiques. Les résultats des analyses phytochimiques et de l'HPLC ont révélé que *M. sativa* contient la plupart des composés actifs, en particulier phénoliques (naringine et acide vanilique) avec une forte activité antioxydante. La caractérisation de CuNPs a confirmé l'implication des molécules biologiques dans la synthèse de CuNPs avec la taille comprise entre 19,8 et 92,8 nm. Les résultats de l'étude in vivo sur les rattes ont montré une variation des paramètres hématologiques et biochimiques chez le groupe des rattes de l'anémie expérimental par rapport au témoin. De plus, les résultats ont illustré un stress oxydatif chez le group PHZ présentant un niveau élevé de MDA et un faible activité de SOD par rapport au groupe témoin. L'histopathologie des tissus osseux, de la rate et rénaux testés a confirmé l'altération de ces tissus avec inflammation, nécrose, hémorragie chez les rats anémiques par rapport au témoin. Cependant, les résultats obtenus montrent une amélioration partielle de toutes les altérations antérieures chez les rats traités par la plante *M. sativa* et CuNPs par rapport aux rattes anémiques. En conclusion, la présente étude suggère que les CuNPs et l'extrait de *M. sativa* pourraient être un effet anti-inflammatoire et antioxydant très prometteur qui peut être utilisé dans un traitement efficace de l'anémie.

Mots clés : Anémie, Phénylhydrazine, *Medicago sativa*, CuNPs, Stress oxydatif, Rattes

الملخص

الملخص

الهدف من هذه الدراسة هو دراسة تأثيرات المستخلص المائي لنبات الفصة (*Medicgo sativa*) وجسيمات النحاس النانوية CuNPs على بعض المعايير البيوكيميائية ومكونات الدم ومعايير الإجهاد التأكسدي عند فقر الدم التجريبي الناجم عن فينيل هيدرازين في إناث جرذان. الدراسة المختبرية التجريبية تمت على 20 أنثى من جرذان ويستار تم تقسيمهم إلى أربع مجموعات، المجموعة الأولى شاهده ، والثانية مصابة بفقر الدم التجريبي (PHZ)، والثالثة مكونة من جرذان مصابة بفقر الدم التجريبي معالجة بجسيمات النحاس النانوية (3 ملغم/كجم) (PHZ + CuNPs) والرابعة مكونة من جرذان مصابة بفقر الدم التجريبي ةمعالج بالمستخلص المائي لنبات الفصة *M. sativa*(200ملغ/كغ) (PHZ + MS). تم تحديد بعض معايير الإجهاد التاكسدي والمعايير البيوكيميائية و معايير مكونات الدم بالاعتماد على الطرق الكلاسيكية في التحليل. تمت دراسة خصائص جزيئات النانو CuNPs بواسطة الطرق التحليلية المعروفة في المختبرات. كشفت نتائج التحليلات الكيميائية للنبات المدروس *M. sativa* أنه يحتوي على معظم المركبات النشطة، وخاصة الفينولات (النارنجين وحمض الفانيليك) مع نشاط عالي كمضادات للأكسدة .أكدت نتائج خصائص جزيئات النحاس النانوية CuNPs مشاركة الجزيئات البيولوجية في تخليق CuNPs بأحجام تتراوح بين 19.8 و 92.8 نانومتر. كما أظهرت نتائج الدراسة على الجرذان تغيرًا في المعايير البيوكيميائية وفي مكونات الدم لمجموعة الجرذان المصابة بفقر الدم مقارنة بالمجموعة الشاهدة. .بالإضافة إلى ذلك ، أظهرت نتائج الإجهاد التأكسدي عند الجرذان PHZ مستويات عالية من MDA ومستويات منخفضة من نشاط SOD مقارنة بالمجموعة الشاهدة. من ناحية اخرى اظهرت اختبرات أنسجة العظام والطحال والأنسجة الكلوية تغييرًا مؤكدًا لهذه الأنسجة مع الالتهاب والنخر والنزيف في الجرذان المصابة بفقر الدم مقارنة بالشواهد. ومع ذلك ،ابانت النتائج التي تم الحصول عليها تحسنًا متباينا في جميع المعايير السابقة عند الجرذان المعالجة بنبات *M. sativa* و CuNPs مقارنة بالجرذان المصابة بالانيميا. في الختام تشير هذه الدراسة إلى أنجزيئات النحاس النانوية CuNPs والمستخلص المائي لنبات *M. sativa* قد يكون لها تأثيرًا طبيا واعدًا للغاية وهذا كمضادًات للالتهابات ومضادًات للأكسدة يمكن استخدامها في علاج فقر

الكلمات الرئيسية : فقر الدم، *Medicago sativa*، جزيئات النحاس النانويه، الإجهاد التأكسدي،الجرذان .

Abbreviation list

Au: gole

BAT: Butyrlated hydroxytolune

BHA: Butyrlated hydoxyanisole

EDTA: ethylenediaminetetraacetate

CAS: Nombre CAS

CAE: gallic acide equivalent

Cu: copper

CuOs: copper oxide

CuNPs: copper oxide nanoparticles

Ca: calcium

CNS: central nervou system

DNA: DeoxyriboNucleic Acid

DPPH: 1,1-diphenyl-2-picryhydrazyl

DM: dry matter

GSH: reduced Glutathione

HGB: hemoglobin

HP: Haptoglobin

ID: iron defivency

IDA: iron defivency anemia

Fe: iron

FRAP: Ferric Reducing Antioxidant Power

FTIR: Fourier-transform infrared spectroscopy

HCT: haematocrit

HGB: haemoglobin

HPLC: High-performance liquid chromatography

IC50: Inhibitory Concentration of 50%

IL-18 : interleukins 18

IL-1 : interleukins 1

IL-6 : interleukins 6

L929: fibroblast

MDA: Determination of malondialdehyde

MCV: mean corpuscular volume

MCH: mean corpuscular hemoglobine

MS: *Medicago sativa*

Mg: magnésium

Mn: menganèse

PSC: pre-school childre

PHZ: phenylhydrazine

PLT: Platelet

PG: propyl gallate

P: phosphorus

RBCs: red blood cell

SEM: Scanning electron microscopy

Si: silicon

SOD : Superoxyde dismutas

TCA: Trichloroacétique

UV-vis: UltraViolet-Visible

WBCs: white blood cells

WRA: women of childearing age

WHO: world health organization

XDR: x-ray diffraction

Zn: Zinc

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Summary

Second Part :Experimental Part

Chapter I: Materials and Methods

Introduction

Introduction

Anemia is a disorder of the blood. Blood is a vital fluid that flows through veins and arteries. Your body contains about 5 to 6 liters of blood, which your heart constantly pumps throughout your body. Blood carries oxygen, nutrients, and other essential compounds. It also helps regulate body temperature, fight infection, and get rid of waste. When something goes wrong with your blood, it can have a huge impact on your health and quality of life(Guzmán Llanos *et al*., 2016) Anemia affects approximately one third of the world's population. Anemia is associated with increased morbidity and mortality in women and children, poor birth outcomes, lower labor productivity in adults, and impaired cognitive and behavioral development in children. Pre-school children (PSC) and women of childbearing age (WRA), (Anémies, 2019)where 8 million Algerians suffer from this disease, which reflects a rate of 20% (Effects, 1995).

For this reason, efforts have focused on developing effective and safe treatments . Plants are a source of bioactive molecules and have been widely used to treat anemia .Alfalfa is a perennial flowering plant belonging to the legume family. It is an important and rich source of vitamins (A, C, E, and K) as well as minerals such as calcium, potassium, phosphorous and iron (Chimique *et al*., 2018).

Herbal experts believe that treating anemia is one of the most important features of this herb because it contains vitamins and minerals (Chimique *et al*., 2018).

It also enhances the absorption of iron from foods, so its effect is evident in reducing fatigue, extreme fatigue, weakness and weakness, and also contributes to relieving shortness of breath and preventing recurrence of infections (Chimique *et al*., 2018).

The ability to process structures and characteristics at the nanoscale level in medicine. It's like having a complex microscopic work through which you can handle cell components. This technology allows the development of biocompatible materials that support cell growth (Boisseau&Loubaton,2011) , Nanoparticles are small materials ranging in size from 1 to 100 nanometres .It has unique physical and chemical characteristics (Lee *et al*., 2011) Due to the widespread application of metal nanoparticles in areas of human connection, Using plant extracts is more useful for biological processes, than others Due to the popularity of copper nanoparticles with their antioxidant activity, inflammation and antibacterial. (Derouiche *et al*,. 2020) For the first time, copper nanoparticles were manufactured using *M .sativa* extract .

In light of these data, the present study was carried out to investigate the two following complementary aspects:

- **The first part:** based on the in-vitro study; extraction of plant extract, preparation of copper nanoparticles, quantitative and qualitative characterization of these compounds and evaluation of their biological property.
- **The second part:** based on the in-vivo study for evaluation of the therapeutic efficiency of alfalfa leave aqueous extract and CuNPs against metabolic, physiological and histological alteration induced by experimental Anemia in rats.

First part

Bibliographic synthesis

Chapter I

Anemia and & phenylhydrazine

1.General characteristics

The WHO defined anemia as being the drop in the hemoglobin level two standard deviations below that of the normal value for the age and gender in question (Guzmán Llanos *et al*., 2016). In practical terms the values of 13 g/dl in men, 12 g/dl in women and 11 in pregnant women can be used. In children aged 6 months to 6 years old 11/g/dl and from 6 years old to 14, 12 g/dl. These criteria are based on studies of the population that do not include seniors over 65, which means that perhaps it cannot be used for elderly people. Anemia is highly prevalent in primary care consultations, pediatrics, and during pregnancy. Iron deficiency (ID) is not the same as iron deficiency anemia (IDA). The latter is the most common cause of anemia in the world and it is a major health problem mainly in underdeveloped countries. The IDA is very widespread, so much so that it affects 5% of children and adolescents, 10% of premenopausal women and 1% of men; and it can affect as many as 40% of the seniors who are looked after at home (Berhe *et al*., 2018).

Anemia occurs if your body makes too few red blood cells (RBCs), destroys too many RBCs, or loses too many RBCs. RBCs contain hemoglobin, a protein that carries oxygen throughout your body. When you don't have enough RBCs of the amount of hemoglobin in your blood is low, your body doesn't get all the oxygen it needs. As a result, you may feel tired or have other symptoms (WHO, 2011).

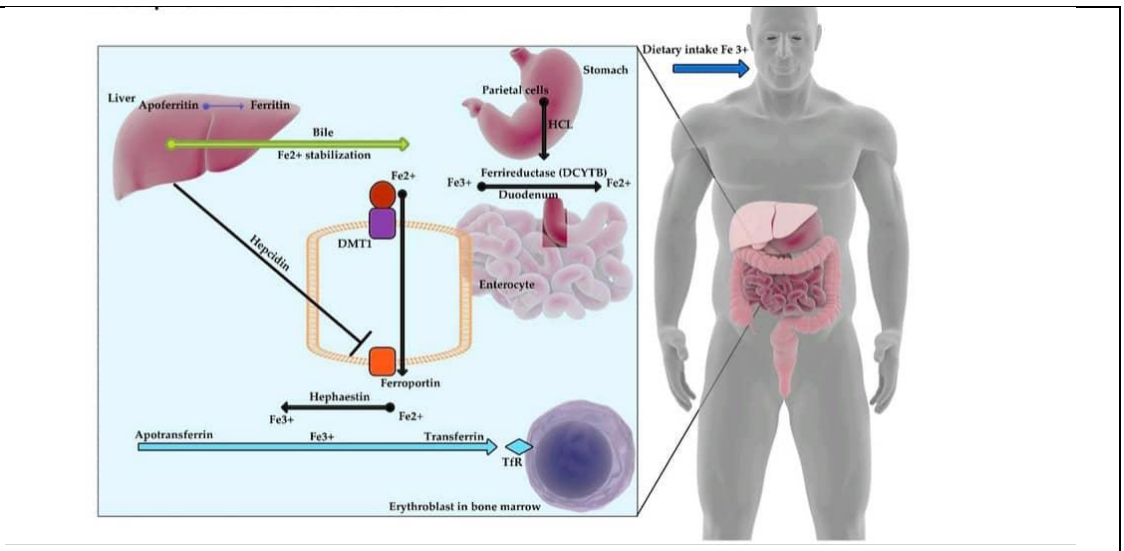

Figure 01: Absorbation and metabolism of iron(Wiciński *et al*., 2020)

1.1.Taxonomy of anemia

A practical way of classifying anemia is by focusing on the red blood cell size (MCV) and the hemoglobin amount per red blood cell (MCH). These are two of the parameters that provide us with the reading of any hemogram using modern cell counters. - The size can point to normocytic anemia, microcytic anemia and macrocytic anemia and according to Hb amount (MCH) normochromic anemia, hypochromic anemia and hyperchromic anemia **(Table 01)** (Guzmán Llanos *et al*., 2016).

Table 01: Classifying anemia according to hematological parameters

	Microcytic	Normocytic	Macrocytic
Hb (g/dl)	H: < 13.5 M: < 12.5 N: < 11.5	M: < 12.5 H: < 13.5 N: < 11.5	H: < 13.5 M: < 12.5 N: < 11.5
HCM (pg)	Hypochromic < 28 pg/h	Normochromic 28-33 pg/h	Hyperchromic ¬ 33 pg/h
MCV (fl)	< 80 fl	80-96 fl	¬ 96 fl

H: Male; M. Female; N. Child; Hb: hemoglobin; MCH: mean corpuscular hemoglobin; MCV: mean corpuscular volume Microcytic anemia and hypochromic anemia are normally caused by iron deficiency anemia.

1.2.Anemia associated with human diseases

There are many factors and causes that lead to anemia, among which we can mention the following resons:

- Problems with how iron is used by the body.
- Not eating enough iron-rich foods.
- Bleeding or blood loss, such as from heavy menstrual periods.
- Pregnancy.
- A lack of folate or B -12 vitamins in the body.
- Treatments for some diseases, such as cancer, that make it harder for the body to make new red blood cells.
- Sickle-cell disease where the body destroys too many red blood cells.
- Immune system problems where the body destroys or cannot make red blood cells.

- Babies less than one year old who drink cow's or goat's milk.
- Babies who are fed formula that does not have extra iron (Anémies, 2019).

In some types of anemia, such as aplastic anemia, your body also doesn't have enough of other types of blood cells, such as white blood cells (WBCs) and platelets(Engel, 2014). WBCs help your body's immune system fight infections. Platelets help your blood clot, which helps stop bleeding. Many diseases, conditions, and other factors can cause anemia. For example, anemia may occur during pregnancy in the body can't meet its increased need for RBCs. Certain autoimmune disorders and other conditions may cause your body to make proteins that destroy your RBCs, which can lead to anemia. Heavy internal or external bleeding—from injuries, for example—may cause anemia because your body loses too many RBCs. The causes of anemia can be acquired or inherited. "Acquired" means you aren't born with the condition, but you develop it. "Inherited" means your parents passed the gene for the condition on to you. Sometimes the cause of anemia is unknown (WHO, 2011).

2.Generalities about phenylhydrazine

Phenylhydrazine (PHZ) was the first hydrazine derivative characterized by Hermann Emil Fischer in 1875. This compound is used worldwide mainly as a chemical intermediate in the pharmaceutical, agrochemical, and chemical industries. PHZ, C6H8N2 (see structural diagram below) has a molecular weight 108; it exists as yellow to pale brown crystals or as a yellowish oily liquid, with a freezing point of 19.6°C and a boiling point of 243.4°C. PHZ metabolism seems to occur via ring hydroxylation and conjugation, excretion is primarily via the urine (Berger, 2007).

Classification :

Synonyms: hydrazinobenzene

Chemical name (CAS): phenylhydrazine

CAS number: 100-63-0

Molecular formula: C6H8N2

Molecular weight: 108.14

Melting point: 20°C

Density at 20°C: 1.099 g/cm3

Vapour pressure at 25°C: 0.13 hPa

1 ml/m3 (ppm) = 4.5 mg/m3 1 mg/m3 = 0.22 ml/m3 (ppm**)** (Effects, 1995).

Structural formula:

Figure 02 : chemical formula of phenylhydtazine(Chimique *et al*., 2018)

2.1 Toxic Effects and Modes of Action

Phenylhydrazine is readily absorbed by the skin and after ingestion and inhalation. It is of high acute toxicity and is a potent methaemoglobin generator. The target organs of the toxic effects are, in addition to the haematopoietic system, also the liver, kidneys and lungs. Phenylhydrazine causes skin and eye irritation and has skin-sensitizing potential.

The substance is genotoxic in vitro and in vivo. The increased incidence of lung tumours and in particular vascular tumours in long-term studies with mice indicates a possible carcinogenic effect.

2.2.Phenylhydazine induced inflammation

Experimental toxicity Acute toxicity Phenylhydrazine is toxic to animals; it induces hemolytic anemia, following the formation of methemoglobin, which results in hepatic, splenic and renal lesions. It is a skin and eye irritant. Subchronic, chronic toxicity Studies on the medium or long-term non-carcinogenic effects of phenylhydrazine are very rare; however, they confirm the powerful haemolytic effect of the product and the functional alterations caused in the liver and kidneys. Genotoxic effects Phenylhydrazine is genotoxic in vitro; in vivo, it causes adducts and fragmentations of mouse hepatic DNA. Carcinogenic Effects Phenylhydrazine,

hydrochloride form, is carcinogenic to mice by the oral route (Hahn *et al*., 2006)The observed haematotoxicity is a result of the reaction of phenylhydrazine with oxygenated haemoglobin to form reactive products, for example oxygen radicals, and methaemoglobin. The radicals cause lipid peroxidation and subsequent membrane damage.

In a recent study it was shown that phenylhydrazine-induced haemolysis causes induction of liver haeme oxygenase, which in turn causes an increase in the bilirubin level in serum. The bilirubin seems to be responsible for lipid peroxidation in the tissues The genotoxic effects are probably the result of DNA methylation, which possibly takes place via intermediary formation of the hydrazone with endogenous formaldehyde.

At least for the genotoxic effects in vitro, other mechanisms such as the formation oforganic radicals in the reaction of oxyhaemoglobin with phenylhydrazine also seem toplay a role (Chimique *et al*., 2018).

3.Oxidative stress

The close association between oxidative stress and lifestyle-related diseases has become well known. Oxidative stress is defined as a "state in which oxidation exceeds the antioxidant systems in the body secondary to a loss of the balance between them." It not only causes hazardous events such as lipid peroxidation and oxidative DNA damage, but also physiologic adaptation phenomena and regulation of intracellular signal transduction. From a clinical standpoint, if biomarkers that reflect the extent of oxidative stress were available, such markers would be useful for physicians to gain an insight into the pathological features of various diseases and assess the efficacy of drugs (Betteridge, 2000).

3.1.Free radicals

A free radical is a chemical species (atom or molecule) which has a single (or unpaired) electron on its external electronic orbitals which gives them a very great instability. Free radicals capable of reacting with different molecules, in particular during chain reactions, the best known example of which is the peroxidation of lipids (Christophe & Christophe, 2011), most free radicals are produced by the mitochondria (Sylvia, 2010). Oxygen is a low-reactive free radical, usually present as a dioxygen. Under physiological conditions 2% to 5% of the oxygen used by the mitochondria is partially reduced by electrons that escape from the respiratory chain carriers, thereby forming more reactive derivatives called reactive oxygen species (ROS) (Ichai *et al*., 2011). These molecules are a family of chemical entities regrouping

oxygenated free radicals (chemical species possessing an unpaired electron) such as the anion superoxide (O•2-), The hydroxyl radical (OH•), Nitrogen monoxide (NO•) and the oxygen derivatives known as active oxygen species (not possessing a single electron) they are not free radicals but they are also reactive and can be precursors of radicals (peroxide anion (O2-2), hydrogen peroxide (H2O2), peroxynitrite (ONOO-) (Lanez & Djouadi, 2015).

3.2. Anti-oxidant systems

We pay attention to two types of antioxidant system : the enzymatic antioxidant system and the nonenzymatic antioxidant system. Antioxidant enzymes include glutathione peroxidase (GR), catalase (CAT) and superoxide dismutase (SOD); while the nonenzymatic antioxidant system are the dietary and endogenous antioxidant chemicals, which include vitamin A, carotenoids, vitamin C, vitamin E, glutathione (GSH), alpha-lipoic acid, coenzyme Q10, L-carnitine and polyphenolic compounds derived from plants, fruits and vegetables (Kedar, 2015; Xuejun *et al*., 2015).

3.3.Oxidative stress and phenylhydazine

Phenylhydrazine and hemedioxide induced heme oxygenase activity in rat liver. This inductive effect was preceded by a decrease in the intrahepatic GSH pool, which occurred several hours before oxygen induction. Administration of α-tocopherol and allopurinol prevented the induction of oxygenase but had no effect on the decrease in GSH levels. These results indicate that the induction of heme oxygenase by phenylhydrazine and diamide is preceded by oxidative stress very likely arising from GSH depletion. Heme-oxygenase induction was not prevented by administration of alpha-tocopherol or allopurinol. Protoporphyrin IX did not affect the molecular pattern of hepatic reductase biliverdin (Kolawole & Dapper, 2017).

Chapter II

Medicago sativa & Copper nanoparticles

1.Generality

Medicinal plants are the Nature's gift to human beings to help them pursue a disease-free healthy life. use of Plants have been used as drugs by since thousands of years ago As a result of accumulated experience from the past generations, today, all the world's cultures have an extensive knowledge of herbal medicine. (Ali Esmail Al-Snafi *et al*., 2021) medicinal plants have been used for treatment of illness and diseases. Ancient Chinese scriptures and Egyptian papyrus hieroglyphics describe medicinal used for plants. Indigenous cultures (e.g. African and American) used herbs in their healing rituals, while others developed traditional medical systems (e.g. Ayurvedic and Chinese medicine) .where recently, the world systems procure more than 80% of their medicaments from plants (Bhavna *et al*., 2007). medicinal plants naturally synthesize and accumulate some secondary metabolites, like alkaloids, sterols, terpenes, flavonoids, saponins, glycosides, cyanogenics, tannins, resins, lactones, quinines, volatile oils etc. which that possess therapeutic properties or exert beneficial pharmacological effects (Ali Esmail Al-Snafi *et al*., 2021).

1.1.Definition of *Medicago sativa*

Medicago sativa Linn. (Leguminosae), commonly known as the "father of all foods" (alfalfa), is a perennial herbaceous leguminous plant species that originated in Asia (Ehsanpour & Razavizadeh, 2005; Duke, 1985; BHMA, 1996). This is the most ancient plant, cultivated throughout the world as a fodder plant. In America, *M. sativa* has been extensively cultivated since the arrival of Europeans. *M. sativa* has been grown for a variety of purposes such as soil improvement, animal feed and medicinal uses (Steppler, 1987). *M. sativa* has a long tradition of use as Ayurvedic and homoeopathic medicine in central nervous and digestive system disorders, and for the treatment of various other ailments. However, only limited research has been conducted on this plant species (FAO, 2006).

1.2.Systematic and taxonomy of *M. sativa*

Fabaceae. Perennial, tall (30) to (110 - 180 cm), hairless. Stems erect, branched. Taproot. (Bafor, 2017).The plant has a taproot which may penetrate deep into the soil, sometimes stretching more than 6 m. Upon germination, a strong taproot develops rapidly and penetrates almost vertically downward. It often reaches a depth of 150-180 cm the first season, 3-4 m by the end of the second year, and may ultimately extend to depths of 6 m or more. However, typically 60-70 percent of the root system is concentrated in the upper 15 cm of soil,

with fibrous roots predominating and bearing most of the nodules. Like other legumes, its root nodules contain bacteria, with the ability to fix nitrogen. To stimulate root growth, the young stand should be irrigated frequently because root development is adversely affected by dryness. Leaves trifoliate, with obovate leaflets or 12 times No oblong, toothed at the top, mucronate. Stipules long-acuminate, toothed at the base. Purple or blue flowers, rarely variegated, white, purple, cream or yellow, grouped in oblong clusters. the fruit is a legume, spiral shaped with 2-6 seeds (De Smet, 1992).

- ✓ **Kingdom**: Plantae
- ✓ **Subkingdom:** Viridiplantae
- ✓ **Infrakingdom:**Streptophyta
- ✓ **Superdivision:** Embryophyta
- ✓ **Division:** Tracheophyta
- ✓ **Subdivision:** Spermatophytina
- ✓ **Class:** Magnoliopsida
- ✓ **Order:** Fabales
- ✓ **Family:** Fabaceae
- ✓ **Genus:** Medicago
- ✓ **Species:** *Medicago sativa*(Al-Snafi, 2015).

Figure 03: *Medicgo sativa*(Undersander *et al*., 2011)

1.3.Desertstribtion of *Medicago sativa*

It is native to Africa (Algeria, Libya, Morocco, Tunisia), Asia (Afghanistan, Cyprus, Iran, Iraq, Palestine, Jordan, Lebanon, Syria, Turkey, Armenia, Azerbaijan, Georgia, Russian Federation, Kazakhstan, Kyrgyzstan, Tajikistan, Turkmenistan, Uzbekistan, Mongolia, China, Korea, Pakistan), Europe (Denmark, Ireland, Norway, Sweden, United Kingdom, Austria, Belgium, Czechoslovakia, Germany, Hungary, Netherlands, Poland, Switzerland, Belarus, Estonia, Latvia, Lithuania, Moldova, Russian Federation-European part, Albania, Bulgaria, Former Yugoslavia, Greece, Italy, Romania, France, Portugal, Spain). It is naturalized in Africa (Egypt, South Africa), Asia (India, Nepal, Sri Lanka), Australasia (Australia, New Zealand),

Europe (Finland), Northern America (United States), Southern America (Brazil, Ecuador, Peru, Argentina,Chile, Uruguay) and it is widely cultivated(FAO, 2006).

M. sativa has a wide range of adaptation and can be grown from very cold northern plains to high mountain valleys, from rich temperate agricultural regions to Mediterranean climates and searing hot deserts (Wrona & Górecki, 2016).

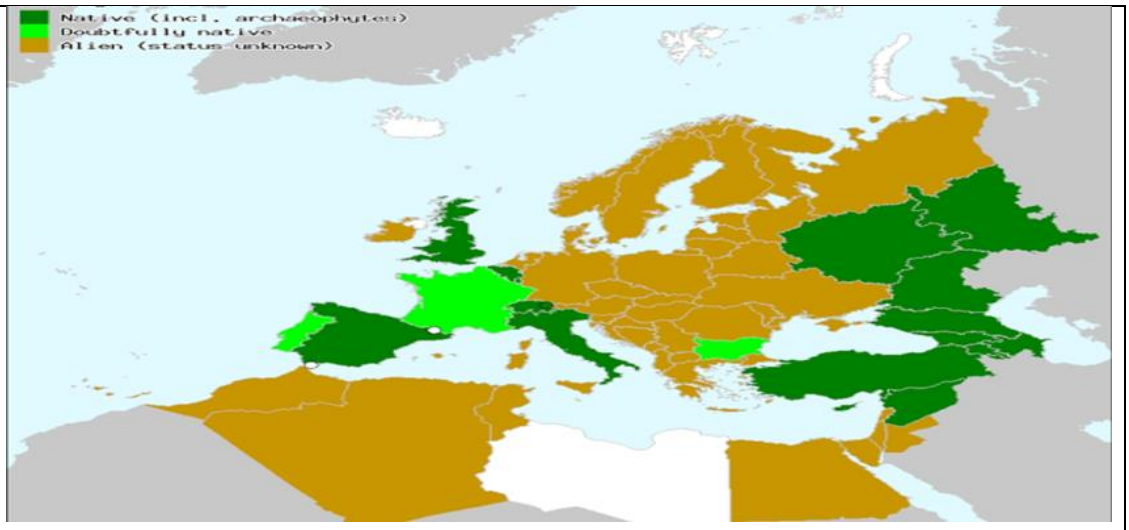

Figure 04: Distribution map of *Medicago sativa* (https://images.app.goo.gl/GmVH7NGmbovG5Quq7)

1.4.Chemical constituents

Alfalfa is rich in essential amino acids such as valine, leucine, threonine and lysine. Moreover, the composition and the ratio of amino acids are considered to be similar to the standard egg white. Its aerial parts are one of the best sources of chlorophyll and vitamins CE, B_1, B_2, B. B2, niacin, folic acid, biotin, inositol. choline, and B-carotene (Stochimal, 2007). It also contains valuable minerals such as Ca, Cu, Fe, Mg. Mn . P. Zn, Si (Zanin, 2009, Bora and Sharma, 2011a, 2011b) Recent studies have demonstrated the presence of lithium (1.12 mg/g) in *M. sativa* (Hanif *et al*., 2015). This mineral is a mood stabilizer and has been widely used in treatment of bipolar disorder.

The pharmacologically active substances present in *Medicago sativa* include alkaloids (stachydrine, homostachydrine), aminoacids (arginine, asparginine, cystine, histidine, isoleucine, leucine, methionine, tryptophan, valine),coumarins (medicagol, sativol, trifoliol, lucernol, 4-o- methyl coumesterol, 3- methoxycoumesterol, 11,12 – dimethoxy -7- hydroxyl coumesterol), flavonoids (quercetin, myricetin, luteolin, apigenin,chrysoeriol, tricin, coumestrol, biochanin A, genistein), (Bora *et al*., 2012) saponins, steroids (stigmasterol, campestrol, cycloartenol, β-sitosterol),acids (lauric, maleic, malic, malonic, myristic, oxalic, palmitic, quinic), vitamins (A, B1, B6, B12, C, D, E, K), ketones (myristone, alfalfone) and other constituents such as fructose, pectin, chlorophyll, minerals and trace elements (Aldo *et al*., 1997).

1.5. *Medicago sativa* extracts efficacies

used in tonic form for the digestive system. It is believed to increase vitality, stimulate appetite and promote weight gain in anorexics. It also reduces constipation, treats chronic ulcers, cures anemia and aids in the treatment of diabetes.

Alfalfa is high in mineral content, and, because of this, it is ideal for bones, joints and skin. It promotes both bone and teeth health. The high chlorophyll content of alfalfa also supports the growth of connective tissue and is beneficial for people suffering from arthritis. It also aids in tissue repair. It is useful to heal wounds, ulcers and abscesses.

Other Uses: Alfalfa is also believed to work in lowering cholesterol. It has been used as an antibacterial and to relieve sinus infections. Because alfalfa is rich in anti-oxidants, it is useful for breaking down toxins in the blood system(Karimi *et al*., 2013)There have even been

reports that the alfalfa plant is useful for prostate and urinary problems (Mikaili & Shayegh, 2011).

1.6.Biological activities

properties alfalfa is a rich source of biologically active compounds - secondary metabolites, showed that total value of phenolics and flavonoids is 37.0 0.02 mg gallic acid equivalent (CAE)g dry matter (DM) and 126+0.17 mg rutin equivalenting DM, respectively. Many studies indicate that some cultivars of alfalfa are also an especially rich source of bioactive saponins (Oleszek., 1996; Tava *et al*., 2011) High biomass production together with the high content of phenolic compounds and saponins make M. sativa a good source of bioactive compounds. Hence, besides its importance as fodder, this species is considered to be a herb beneficial to the human body, Saponins and flavonoids are valuable components of animal and human diet. and have many pharmaceutical applications (Gholami *et al*., 2014; Bora and Sharma, 2011a, 2011b: Glowniak *et al*., 2007). This plant has been used for centuries in traditional medicine to improve memory, to cure kidney problems, asthma, cough, arthritis and central nervous system disorders (finkler, 1985;Inamul, 2004), Contemporary studies indicate that extracts rich in saponins are effective in lowering blood cholesterol levels. Not without.

1.7.Other Biological activities

1.7.1.Antimicrobial effects

Medicago sativa (Leguminosae), which is one of the most reputed medicinal plant traditionally used antimicrobial (Bhattarai *et al*., 2021). where The antimicrobial effect of aqueous extract of alfalfa seed was studied against Pseudomonas aeruginosa, Escherichia coli , Staphylococcus aureusaures. it showed moderate activity against Gram positive (Ali Esmail Al-Snafi *et al*., 2021).

M. sativa plant extracts rich in saponins showed strong antifungal potential to successfully check the growth of Candida albicans along with certain clinical pathogenic fungal strains mainly by inhibiting the germ tube formation, retarded the growth of fungal hyphae, and lessened the adherence of yeast cells and eradication of biofilm development at 24 hours after treatment .It is further stated that saponins extracts of *M. sativa* in a dosage range harmful to check the growth of fungiare least toxic to the mice fibroblast L929 cells, which showed them being safe to use for human antifungal conditions (Sadowska *et al*., 2014).

1.7.2.Antioxidant Potential

.the extraction studies reported that *M. sativa* plants extracts bear strong antioxidant potential. For instance, various parts (roots, stem, leaves) of *M. sativa* plant ethanolic extracts yield various phenolics, flavonoids, and saponins, all of which show higher antioxidant potential (Krakowska *et al*., 2017).

A positive correlation was observed between the phytochemicals content and the strong biological activities such as antioxidant activity, anti-inflammatory (Barros *et al*., 2007). This investigation for natural antioxidants with the plant origin is also being explored asan alternative to the synthetic antioxidants, such as butylated hydroxyanisole (BHA), propyl gallate (PG) and butylated hydroxytoluene (BHT), used in food and pharmaceutical industries (Brunet *et al*., 2009) as the synthetic antioxidants may possess some side effects and toxic properties such as carcinogenic to human health (Manian *et al*., 2008).

diphenyl-1-picrylhydrazyl (DPPH) indicated a steady increase in the scavenging activity of free radicals in the extract and the standards in the range of 0 to 250 μg/ml.

The antioxidant activity of Alfalfa by DPPH method showed 54.42% inhibition of free radicals at the concentration of 250 μg/ml of Alfalfa crude extract, while this value for the Vitamin E and C were 90.25 and 91.06%, respectively (Karimi *et al*., 2013).

In the treatment of anemia

Found that the leaf concentrate is effective, and more palatable, alternative to Fe and folic acid supplements for treating anemia in adolescent girls (Vyas *et al*., 2009).

1.8.Effcacy in diseases

In recent years, considerable interest has been generated on a leguminous plant *Medicago sativa* (MS) (Leguminosae), which is one of the most reputed medicinal plant traditionally used to improve the memory, to cure kidney pain, cough, sore muscles, as rejuvenator, ant diabetic, antioxidant, anti-inflammatory, antimicrobial and in CNS disorders .Moreover, MS has a long tradition of use as ayurvedic and homoeopathic medicine in CNS disorder. Phytochemical reports on MS indicate that the plant contains flavonoids, alkaloids, phytoestrogens, coumarins, digestive enzymes, triterpenes, saponins, and phytosterols , .Several clinical and animal studies indicate that the ingestion of *M. sativa* reduces cholesterol absorption and atherosclerotic plaque formation in the arteries ,*M. sativa* is beneficial in

cardiovascular complaints ,convalescence and debility ,diabetes and also when used as a tonic after blood loss and during anemia . The plant has been shown to have anti-tumor activity against certain types of leukemia cells in mice and selective toxicity in dog cancer cells grown in vitro (Bora & Sharma, 2011).

seeds extract of *Medicago sativa* possesses anxiolytic activity in mice which may elicit anxiety by inhibiting GABA ergic mechanisms31. The results show that the *Medicago sativa* protected significant number of animals against anxiety by this model. Some natural and synthetic flavonoids have been found to bind specifically and competitively to benzodiazepine receptors and exhibit anxiolytic effects in the EPM test in rodents. Synthetic flavonoids like 6-bro-moflavanone, 5-methoxy-6, 8-dibromoflavanone and 6-bromo-3-nitroflavone possess anxiolytic-like properties31. Natural flavonoids like apigenin, 6-methyl apigenin, chrysin, hesperidin, luteolin, orientin, isoorientin and wogonin have been reported to possess anti-anxiety activity. 32,33. Thus, flavonoids constituents present in the plant may be responsible for its noted anxiolytic activity (Info, 2014).

1.9. Toxicity Doses

Moderate consumption of *Medicago sativa* leaves in teas and capsules is generally considered safe and without significant side effects. Aggravation of lupus, or promotion of lupus-like symptoms have been reported from the ingestion of large amounts of *Medicago sativa* seeds and sprouts, an action attributed to the amino acid, canavanine (Ali Esmail Al-Snafi *et al*., 2021**)**.

2.Copper nanoparticles

2.1.Nanotechnology

The term " Nano " comes from the Greek word dwarf which reveals the particle size to lie in the range of 1 to 100 nm. (Kiflom Gebremedhn *et al*., 2019) Nanotechnology technology to manipulate and control a substance at the nanometer (nm) level (1 nm = one billionth of a meter . The nanometer level is the level of atoms and molecules (Yokoyama, 2009). New devices have been created with fascinating functions making the best use of the special properties of nano sized substances.

This has resulted from the convergence chemistry , physics , biology and engineering to form the new field of nanotechnology(Tegart, 2003).

2.2.Nanoparticles

In recent years , the development of metal and metal oxide NPs has greatly enhanced the biomedical field in terms of biosensing , imaging , diagnosis , and therapy . The most commonly used metals and their oxides are gold (Au) , silver (Ag) , and copper (Cu). (Letchumanan *et al*., 2021) . nanoparticles , due to their unique physical and chemical properties and the low of preparation , have been of great interest recently (Derouiche *et al*., 2020).Additionally , Cu NPs are efficient catalysts , with high yields and easy product separation , and they can be reused repeatedly . Cu - free ions are potentially harmful to the human body at the cell , organ , and body levels . Therefore , Cu ions in living organisms should be regulated . Cu NPs can be easily oxidized to form copper oxides (CuOs) , which are inorganic NPs . Both Cu and CuO NPs are used extensively as anticancer (Ouidad *et al*., 2020)experiments have shown anti-inflammatory , anti - bacterial and oxidative stress protective effect (Derouiche *et al*., 2020) . This is mainly due to the fact that NPs are able to interact with the biological system at cellular levels for various reactions and functions. (Ouidad *et al*., 2020) .CuO NPs are applied in areas such as biocidal activity , antioxidant , antibacterial , magnetic phase transitions , gas sensors , catalysis , and superconductivity (Kiflom Gebremedhn *et al*., 2019).

2.3.Green synthesis

Green synthesis of Cu / CuO - NPs by plant extracts The environmentally accepted ' green chemistry ' idea has been applied to the biosynthesis of nanoparticles for the creation of clean and environmentally friendly nanoparticles , which incorporates bacteria , fungi , plants , actinomycetes , and other organisms , and is referred to as ' green synthesis ' . Biosynthesis of nanoparticles utilizing the organisms mentioned above exemplifies a green alternative for the creation of nanoparticles with novel characteristics (Akintelu *et al*., 2020). Unicellular and multicellular organisms are permitted to respond these syntheses . , for the mentioned reason , Copper oxide nanoparticles have been widely synthesized using various plant extracts . In this plant - based manufacturing process , the metal salt is mixed with the plant extracts and the reaction takes 1-3 h to complete at room temperature . Plant extracts include a variety of bio active metabolites , including flavonoids , phenols , proteins , terpenoids , and tannins , which serve as reducing and stabilizing agents , transforming metallic ions into nanoparticles (Letchumanan *et al*.,2021) . The plant extract produces electrons , which cause copper salts to get reduced . Copper oxide nanoparticles are formed when phytochemicals react with copper ions , resulting in reduction (Ouidad *et al*., 2020).And in the SEM analysis the CuNPs image

was identified. which were bio-synthesized by plant extract, the nanoparticle has a spherical shape with a size of less than 90 nm (Derouiche *et al*., 2020).

Second Part

Experimental Part

Chapter I

Materials and Methods

1.Materiel

1.1. Plant materials

In this study, (*Medicago sativa)* were obtained from the market. These herbs were powdered by mechanical grinder until a fine powder was obtained. The powders of *Medicago sativa* stored at room temperature in airtight containers protected from bright light until the beginning of the experiment.

Figure 05: Leave *Medicago sativa* (original photo)

1.1.1. Aqueous extract preparation

The aqueous extract was prepare by adding 500 ml of distilled water to 50 g dry powder of plant at 50°C during 2 hours. After 24 h of maceration at room temperature the mixture was filtered by Whatman paperthen evaporated by using rotary evaporator (Derouiche *et al*., 2019).

1.1.2. HPLC fractionation and analysis

Extract of *Medicago sativa* (10mg) were fractionated by CTO-20AC model HPLC with a photodiode array detector (HPLC-DAD) (pf 425–250 Interchim, C8 column Zorbax, 150 × 21.5 mm (250 bars)) at a concentration of 100 g/L. The mobile phase consisted of methanol (A) and water (B) with the following elution gradient: 0 min 0% A 100% B, 26.0 min 100% A 0% B; 26.1–29.4 0% A 100% B, 29.4–29.5 0% A 100% B. The flow rate was 20mL/min and the column temperature were set at 25 °C. Chemical characterization of *Medicago sativa* extract was carried out by comparing the detected polyphenol peaks with respect to retention times with those of standard chemicals (such as chlorogenic acid, caffiec acid,vanillic acid, quercetin, Naringin, Rutin, Vanilin, p-Coumaric acid, Gallic acid) that were monitored at 250 nm using the same HPLC system. All standards were purchased from Extrasynthese or Sigma-Aldrich. Identity and purity of the chemical standards were assessed by HPLC analysis.

1.2.Green synthesis of copper oxide CuO nanoparticles

For the biosynthesis of phyto-copper nanoparticles, the suitable reaction mixture was prepared by adding 2g of the copper sulfate was added into the defined amount of the prepared walnut aqueous extract (20 ml) and the reaction solutions were mixed using a heater-stirred. Both flasks were incubated for 1:30 -2h in the rotary shaker under normal conditions at 60°C,the samples were put in an electric furance memmert adjusted at 200°C for 2 h . Later, the synthesized phyto-copper nanoparticle (CuNPs) were separated and purified by continuous centrifugation (3900 rpm; 10 min; 70°C) With double distilled water and ethanol. The dried CuNPs were kept at 60°C for further characterization and bioactivity study.

1.3. Characterization of copper nanoparticles

1.3.1. UV-Visible Spectroscopy

Synthesis of copper nanoparticles solution with *Medicago sativa* may be easily observed by ultraviolet-visible (UV-Vis) spectroscopy. The bio-reduction of the cu+ions in solutions was monitored by periodic sampling of aliquots (1 mL) of the aqueous component and measuring the UV-Vis spectra of the solution. UV-Vis spectra of these aliquots were monitored as a function of time of reaction on a 6705 UV-Vis spectrophotometer JENWAY in 200-700 nm range operated at are solution of 1 nm.

1.3.2. Scanning electron microscope (SEM) and energy dispersive X-ray (EDX)

SEM is a type of electron microscope that images a sample by scanning it with a high energy beam of electrons in araster scan patterns. SEM and EDX analysis were done using VERTIV-MODEL 6390 machine. Thin films of the sample were prepared on a carboncoeted copper grid by just dropping a very small amount of the sample on the grid, extra solution was removed using a blotting paper and then the films on the SEM grid were allowed to dry by putting it under a mercury lamp for 5 min.

1.3.3. X-ray diffraction (XRD) analysis

The particle size and nature of the copper nanoparticle were determined using XRD. This was carried out using Shimadzu XRD-6000/6100 model with 30 kv, 30 mA with Cuk a radian at 20 angles. X-ray powder diffraction is a rapid analytical technique primarily used for phase identification of a crystalline material and can provide information on unit cell dimensions. The analyzed material is finely ground, and average bulk composition is determined. The particle or grain size of the particles on the copper nanoparticles was determined using Debye Scherrer's equation. $D=0.94\lambda\times B^{\cos}\theta$.

1.3.4. FTIR spectroscopy

To determine the biomolecules, present in the extract, FTIR analysis was carried out for the reduction of cu ions with the spectral range of 400-4000 cm-1. Here the sample was centrifuged at 3900 rpm for 10 min dried using hot air oven and ground with KBr to form apellet. Then, the pellet was analyzed using Cary 630 model FTIR instrument.

1.4. Animal Materials

1.4.1. Animal care

In this study, 20 female Wister rats aged 5 weeks old and weighting 150 -177 g were obtained at the Animal Service of the Pasteur Institute, Algeria. The animals were carried under the same conditions, of and an ambient temperature of (25 ±16) C° for ten days. Animals have free access to water and food containing sugar and oil (Southon *et al*, 1984), The experiment was conducted over a period of 21 days. After a period of adaptation, the animals were divided into four experimental groups of 5 animals eachas follows:

1.4.2 . Treatment animals

- ✓ **Group 1(**Control groups): Healthy rats received distilled water.
- ✓ **Group 2**(Exp Anemia): Experimental Anemia rats (by injecting phenylhydrazine at dose 40mg/kg).
- ✓ **Group 3** (EA +CuNPs): Experimental Anemia rats treated orally by phyto-copper nanoparticles (3mg/Kg b. w/w K).
- ✓ **Group 4** (EA + *M. sativa*):Experimental Anemia rates treated orally by aqueous extract of *M. sativa* (200Mg/Kg b. w/w K).

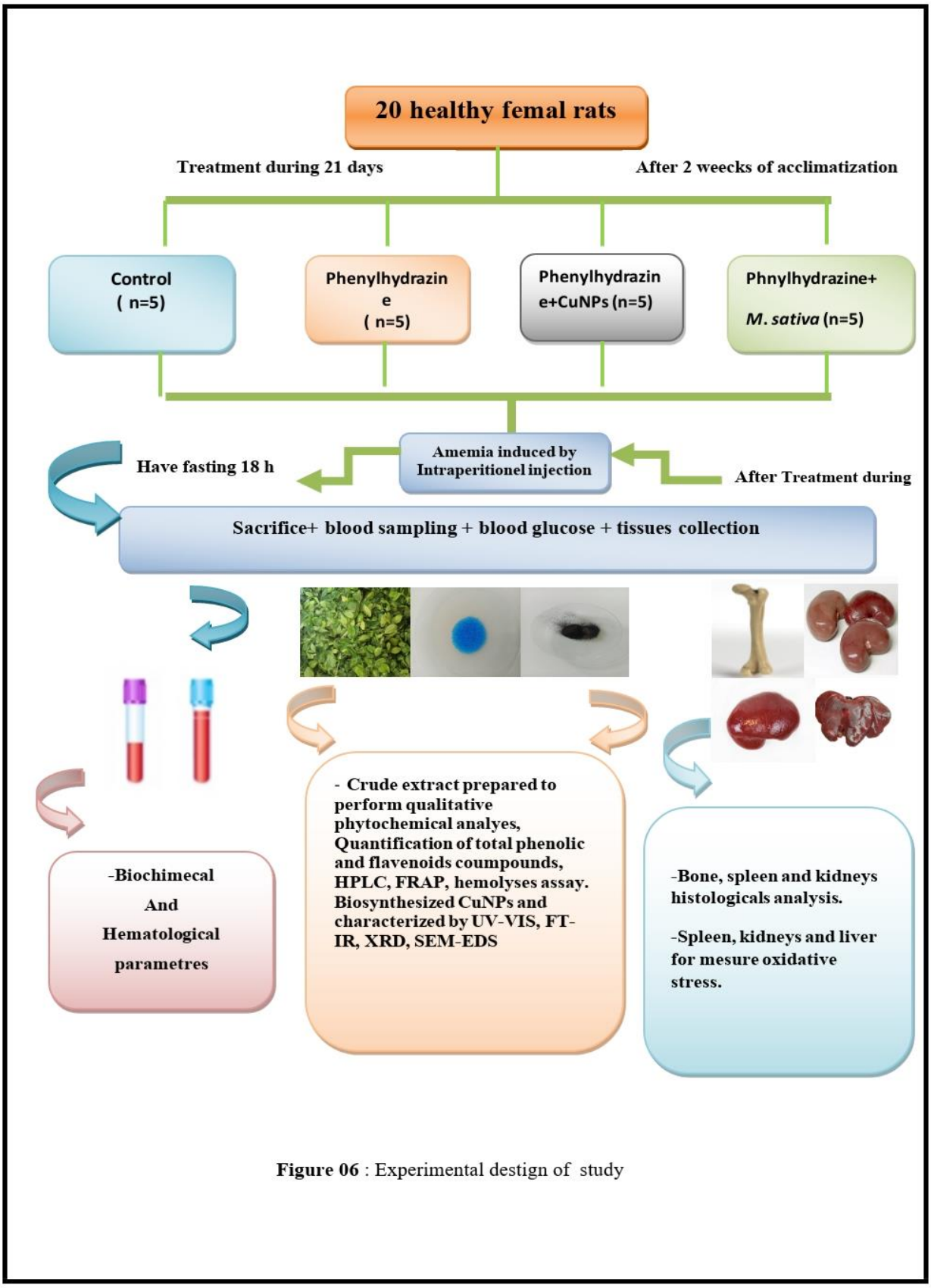

Figure 06 : Experimental destign of study

1.4.3. Sacrifice, blood and tissues collection

After 12 hours of fasting, these animals were sacrificed under slight anesthesia by chloroform (94%) by inhalation; blood samples were collected during the slaughter of animals into EDTA tube to carried FNS and dry tubes. The serum was obtained by centrifugation for 10 min at 3000 tour/min and used for biochemical analysis assays; blood sugar level measured during rat's slaughter using glucometer. Then the liver, kidneys, spleen and bone were isolated from these animals and washed in normal saline. Then it was laid flat and the number and degree of erosions were counted and scored Liver, spleen, bone and college stored at -20 C for oxidative stress, also A piece of the kidneys and spleen And bone of each group of these animals were taken and placed in 10% formaldehyde for histological analysis.

1.4.4.Reagents and products

Sodium Chloride (Nacl), Methanol, Chloroform, Phosphoric Acid (H3PO4), Bovine Serum Albumin (BSA), Gallic Acid, Trichloroacetic Acid (TCA), Thiobar Bituric Acid (TBA), Butylated Hydroxy Toluene (BHT), Chloride Hydrogen HCl, Tris, Salicylic acid, DTNB (5-5'-dithiobis2-nitrobenzoic acid), hydrogen peroxide (H2O2), FeCl3, Fehling liquor, sulfuric acid. Aluminum chloride (AlCl3),salicylic acid .

2. Methods

2.1. Phytochemical analysis

The phytochemical analysis was carried out on the aqueous extracts prepared from the plant by qualitative characterization method according to (Evans, 2009; Harborne, 1998; Wadood *et al*., 2013 & Harborne, 1973).

2.1.1. Phenols

Introduce 5 ml of extract in a test tube and drops few of natural 5% ferric chloride solution. A dark green color indicates the presence of phenolic compounds.

2.1.2. Flavonoids

In a test tube, introduce 5ml of extract, 5ml of diluted ammoniac and 1ml of H_2SO_4. The appearance of a yellow color indicates the presence of flavonoids.

2.1.3. Alkaloids

1 ml of aqueous extract were treated with a few drops of hydrochloric acid then 1–3 drops of Wagner reagent were added. The appearance of brown precipitate reveals the presence of alkaloids in the sample.

2.1.4. Tannins

In a test tube, introduce 5 ml of extract and add 1 ml of a 2% aqueous solution of ferric chloride (FeCl3). The presence of tannins was indicated by a greenish or bluish-blackish coloration.

2.1.5. Terpenoids

The formation of a reddish-brown color indicates the presence of terpenoids, through the addition of chloroform (2ml) and concentrated sulfuric acid (3 ml) to 5 ml of plant extract.

2.1.6. Reducing compound

Add Fehling's liquor (1ml of reagent A and 1ml of reagent B) to the extract and incubate the whole in a boiling water bath, the appearance of a brick-red precipitate indicates the presence of reducing sugars.

2.1.7. Saponins

In a test tube, introduce 5ml of extract, mixed with 5ml of distilled and with vigorous manual agitation. The formation of a steady foam indicates the presence of saponins.

2.1.8. Steroids

For 1ml of plant extract, add 0.5ml of acetic acid solution, followed by 0.5ml of concentrated H2SO4. If the solution does not give any green color, it proves the presence of unsaturated steroids. In a second tube, the same volume of H2SO4 was added. The presence of the red color indicates the presence of steroid derivatives.

2.2. Total phenols and flavonoids compounds

2.2.1. Total phenols

Determination of the total polyphenols was carried out according to the Folin-Ciocalteu (FC) method (Boizot & Charpentier, 2006): 100 µl of artichoke extract are mixed with 500 µl of the FC reagent and 400 µl of Na_2CO_3 at 7.5% (w / v). The mixture is stirred and incubated in the dark and at room temperature for ten minutes and the absorbance is measured at 760 nm by a UV spectrophotometer. The results are expressed in mg gallic acid equivalent/ g of dry vegetable material with reference to the calibration curve of gallic acid. Calibration curve is carried out by gallic acid at different concentrations (20 - 40 - 60 - 80 - 100 - 120 µg/ml) under the same conditions and the same steps of the assay. The results are thus expressed in milligrams of gallic acid per gram of dry extract (mg of EAG / g). All measurements are repeated 3 times.

2.2.2. Total flavonoids

The determination of total flavonoids was carried out according to the method described by (Dehpour *et al*., 2009): 500 µl of each extract, 100 µl $AlCl_3$, 100 µl of 1 M sodium acetate and 2.8 ml of distilled water. The mixture is stirred and then incubated in the dark and at room temperature for 30 minutes. The blank is made by replacing the extract with 95% methanol and

the absorbance is measured at 415 nm using a UV spectrophotometer. The results are expressed in mg equivalent quercetin / g of dry vegetable material with reference to the quercetin calibration curve. The quercetin calibration curve is performed by quercetin at different concentrations (20 - 40 - 60 - 80 - 100 – 120 µg/ml) under the same conditions and the same steps of the assay.

2.3. Antioxidant activity

Take 500µl of sample and Add 1.25ml of the buffer solution (0.2 M, PH = 6.6). Add to 1.25 potassium fericianure.Then Incubation during 20 min in a water bath at 50 ° C. After cooling, add 1.25ml of the aqueous TCA solution (10%) to stop the reaction. Centrifugation at 3000 rpm for 5 minutes. Then take1.25 ml of supernatant are then mixed with 1.25 ml distilled water and 250 µl FeCl3 (0.1%). The absorbance was measured at700 nm against a blank.The results expired by IC50, after calculating of the inhibition percentage values according to(Yazdani *et al*., 2019) as follows:

$$\mathbf{IP\ (\%) = 100 - \frac{OD\ controle}{OD\ sample} \times 100}$$

2.4. Hemolysis assay

Hemolysis assay was done as described by (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 ºC. 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 µL of 10 erythrocytes were stored at 40C and used within 6 h for the hemolysis assay. 50 µL of 10 dilutions (100 uL Erythrocytes suspension: 900 µL 1XPBS) of erythrocytes suspension was mixed with 100 µL of test samples *Medicago sativa* (48µg/mL), 100 µL of 1XPBS was used active control and 100 uL of 1% SDS as positive controls. Reaction mixture was incubated at 37ºC water bath for 60 min. Volume of reaction mixture was made up to 1 mL by adding 850 µL of 1XPB. 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:

$$\text{Hemolysis inhibition (\%)} = 100[\text{Abs Sample} \div \text{Abs Control}] \quad 100$$

2.5. Oxidative stress parameters

2.5.1. Preparation of homogenates

About 1g of liver was homogenized in 9ml of buffer solution of Tris buffer saline (TBS, pH=7.4) while 0.5g of kidneys and spleen was homogenized in 9ml of buffer solution. Homogenates were centrifuged at 3900rpm for 20 min and the obtained supernatant was used for the determination of antioxidant activity.

2.5.2. Determination of Malondialdehyde (MDA) level

MDA was measured according to the method described by Sastre *et al* ., (2000). In brief, Pipette 300 µl of sample, 1200 µl of TBA reagent into the glass and screw test tubes and seal. Heat the mixture in the Marie bath at 100 °C for 15 minutes. Then cool in a coldwater bath for 30 minutes leaving the tubes open to allow evacuation of the gases formed during the reaction. Centrifuge at 3000 rpm for 5 minutes and read the absorbance of the supernatant at 532 nm using a spectrophotometer. TBARS concentration was determined using the MDA molecular extinction coefficient (å= 1,53 105 M-1 cm-1). The results were expressed in µmol / mg proteins.

2.5.3. Determination of Super Oxide Dismutase (SOD) activity Procedure

The assay method of SOD activity using the NBT by the superoxide anion ($O_2^{\cdot -}$), is used as a basis for detecting of presence of SOD by measuring the spectrophotometrically absorbance at 560 nm (Beauchamp & Fridovich, 1971).

Operation mode

Collect in tubes	Blank	Sample
EDTA-Met (0.1mM, 13mM)	1000µL	1000µL
Phosphate buffer (50Mm)	1800µL	1800µL
Sample	-	50
Phosphate buffer (50Mm)	1000µL	950µl
NBT (75µM)	100µL	100µL
Riboflavin (2µM)	50µL	50µL

Expression of results

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

Inhibition percentage of NBT reduction by SOD

2.6. Histopathological study of spleen, bone and kidneys tissues

After rats sacrificed, spleen, kidneys and bone tissues were removed and immersed in fixative (solution 36% formaldehyde) intel the time of slices preparation. Whish dehydrated in ascending graded series of ethanol, cleaned with toluene, immersed in paraffin, and colored with hematoxylin and eosin. Histopathological evaluation was performed with light microscope.

2.7. Statistical analysis

Our statistical study is carried out by the Minitab software using (Student t test) to compere means among our different experimental groups; Differences were considered statically significant at $p<0.05$.

Chapter II

Results & Discussion

1. Results

1.1. In vitro essays of *Medicago sativa*

1.1.1. Qualitative phytochemical analysis

Results of phytochemical essays shows that aqueous extract of *M. sativa* rich on different important chemical compounds such as flavonoïds, phenols, saponins, and terpenoïds but our extract plant is poured from alkaloids Reducing compound.

Table 02: Qualitative Phytochemical content in essays for aqueous extract of *M. sativa*

Compounds	Alkaloids	Flavonoïds	Terpenoids	Phenols	Tannins	Reducing compound	Saponins
Aqueous extract *M. sativa*	–	+	+	+	–	–	+

(+): Present, (-): Absent

1.1.2. Dosage of polyphenols

Phenols and flavonoid compounds

The results presented in table 03 showed a richness from each of total phenols and flavonoids in *M. sativa* aqueous extract.

Table 03: Total Phenols and Flavonoïds concentration in aqueous extract of *M. sativa*

Compounds	Polyphenols (Mg of GAEq/g of extract)	Flavonoïds (Mg QEq/g of extract)
Aqueous Extract of *M. sativa*	322.15 ± 22	271.3 ± 17.9

1.1.3. HPLC analysis of aqueous extract of *M. sativa*

different number of phenolic compounds (chlorogenic acid, vanillic acid, p.coumaric acid, Naringin and Rutin)and different concentrations (chlorogenic acid 1.50 µg/mL, vanillic acid 2.43 µg/mL, p.coumaric acid 0.30 µg/mL, Rutin 3.87µg/mL), then Naringin with concentration(12.21µg/mL).

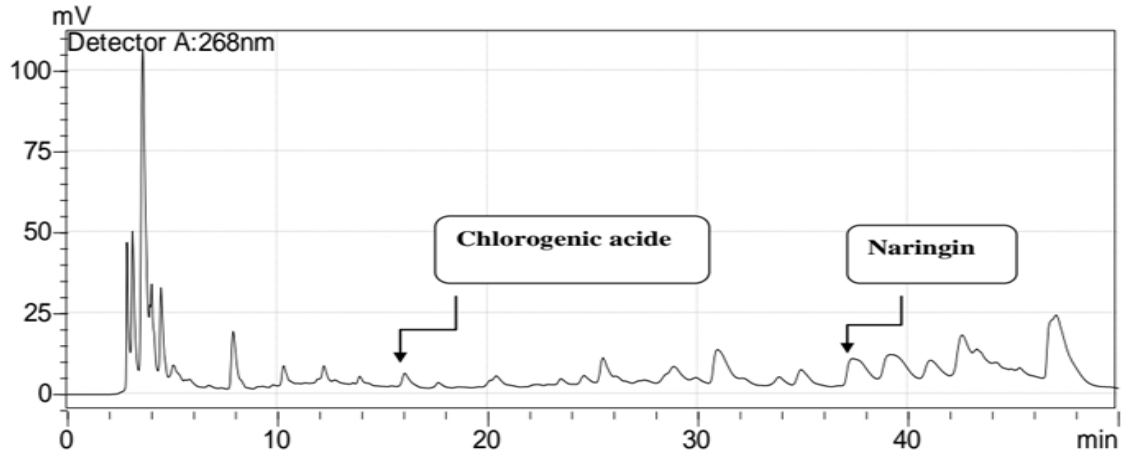

Figure 07: HPLC chromatogram of the aqueous extract of *M. Sativa*

Table 04: Chromatographic charactreateristics of different polyphenolic constituents of *M. sativa*

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.825	256852	44531	1.865	8.223
2	3.080	667633	55185	4.848	10.190
3	3.561	1667731	111292	12.111	20.550
4	3.954	484181	33295	3.516	6.148
5	4.427	436827	32241	3.172	5.953
6	4.995	264021	9604	1.917	1.773
7	5.564	161542	4058	1.173	0.749
8	6.588	76390	2277	0.555	0.420
9	7.320	32303	1322	0.235	0.244
10	7.929	381526	18645	2.771	3.443
11	9.054	30954	1429	0.225	0.264
12	9.301	23247	1493	0.169	0.276
13	9.736	47089	1889	0.342	0.349
14	10.218	200087	7755	1.453	1.432
15	10.942	34202	2157	0.248	0.398
16	11.323	47301	2312	0.343	0.427
17	11.624	35956	2188	0.261	0.404

Peak#	Ret. Time	Area	Height	Area %	Height %
18	11.883	45222	3422	0.328	0.632
19	12.197	171253	7498	1.244	1.385
20	12.711	96854	2768	0.703	0.511
21	13.632	32545	2066	0.236	0.382
22	13.915	138984	4140	1.009	0.764
23	15.117	13324	916	0.097	0.169
24	15.535	158185	5193	1.149	0.959
25	17.693	40269	1688	0.292	0.312
26	18.402	9788	343	0.071	0.063
27	18.983	2726	147	0.020	0.027
28	19.928	143374	3571	1.041	0.659
29	20.978	1228	99	0.009	0.018
30	21.745	3260	180	0.024	0.033
31	22.192	10252	551	0.074	0.102
32	22.464	9756	621	0.071	0.115
33	23.160	97707	3145	0.710	0.581
34	23.760	14544	873	0.106	0.161
35	24.312	134979	3429	0.980	0.633
36	25.270	351604	8837	2.553	1.632
37	26.448	90169	2464	0.655	0.455
38	27.117	14410	1119	0.105	0.207
39	27.699	161793	6822	1.175	1.260
40	27.897	173378	6663	1.259	1.230
41	28.624	109115	3770	0.792	0.696
42	29.110	39690	2288	0.288	0.422
43	29.674	767345	12929	5.572	2.387
44	32.127	9921	249	0.072	0.046
45	33.537	122753	3103	0.891	0.573
46	34.567	236712	6236	1.719	1.151
47	35.663	688308	11183	4.998	2.065
48	37.535	898661	11913	6.526	2.200
49	39.573	552946	6757	4.015	1.248
50	41.738	492460	9268	3.576	1.711
51	42.692	358266	8960	2.602	1.654
52	43.278	196080	9045	1.424	1.670
53	43.491	206976	8520	1.503	1.573
54	43.923	193059	7538	1.402	1.392
55	44.342	310327	6928	2.254	1.279
56	45.354	134753	6097	0.979	1.126
57	45.910	1689705	28555	12.270	5.273
Total		13770523	541568	100.000	100.000

1.1.4. Antioxidant activity

The results of the FRAP assay are reported in Figure 14. The greatest inhibitory activity observed was in the case of Vitamin C, reaching as high as 90.68% at 0.7 mg/mL, while for *M. sativa* a concentration as 93.85% at 0.8 mg/ml and for Cu NPs a concentration as98.63% at 0.8 mg/ml .The concentration of aqueous extract of *M. sativa* resulting in a 50% inhibition of the free radical, IC50, was 17.32µg/mL. IC50 values with regression coefficients (R^2=0.99), for aqueous extract of *M. sativa*. The standard Vitamin C had IC50 values of 4.12 µg/mL with high regression.

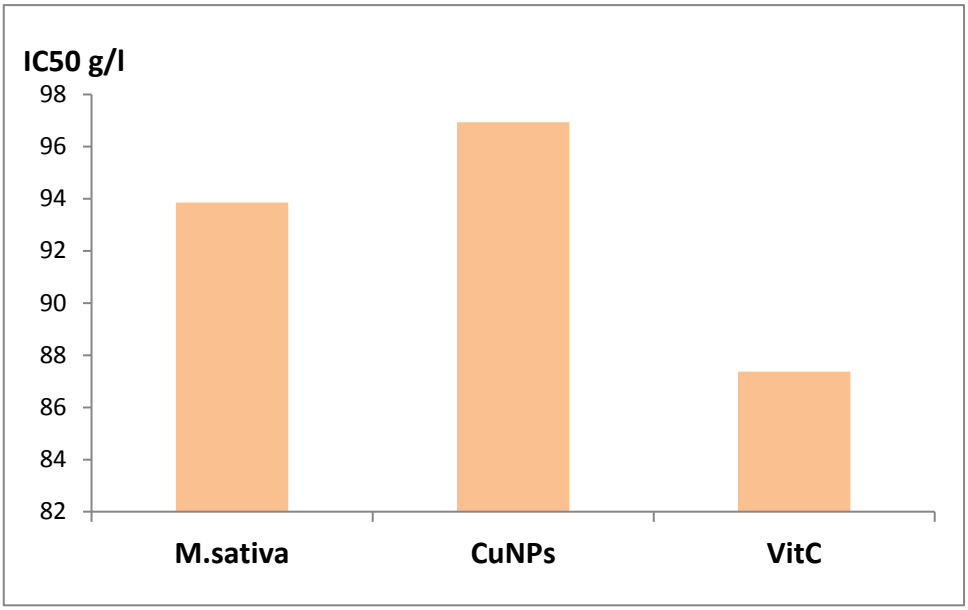

Figure 08: FRAP assay for aqueous extract of and *M. sativa* and CuNPs and Vitamin C

1.1.5. Hemolysis assay

Dehydration and delayed proton equilibria of human blood erythrocyte membrane mediated by phosphate buffer (XPBS, pH= 7.4) induces membrane damage and subsequently hemolysis. The antihemolytic activity of the various Concentration(0.2-1mg/mL) of aqueous extract of *M. sativa* and CuNPs on human blood erythrocytes are presented in **Fig 07**. At the concentration 1mg/mL of aqueous extract of *M. sativa* showed maximal antihemolysis activity (8.60%) in other the maximal antihemolysis activity of CuNPs showed (91.11%) at concentration 1mg/mL.

Interestingly, at various concentrations of CuNPs , the lower the concentration, the greater of antihemolysis activity. On the other hand, we notice the exact opposite, at various concentrations of aqueous extract of *M. sativa*, the higher the concentration, the higher of antihemolysis activity. antihemolysis activity with high regression coefficient (R^2 =0.9184) (for aqueous extract of *M. sativa*) and in CuNPs with regression (R^2 =0.5692).

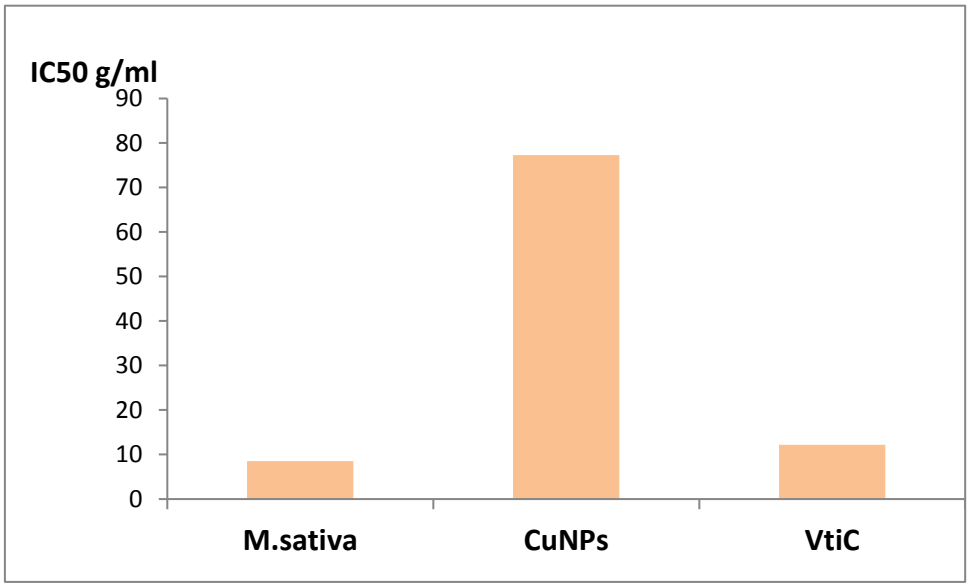

Figure 09: Antihemolytic activity of *aqueous M. sativa* extract and CuNPs.

1.2. Characterization of copper nanoparticles

1.2.1. Formation of copper Nanoparticles (CuNPs)

Copper is blue **(Fig.10.A)** and extract of *Medicago sativa* is dark green in color. After adding *Medicago sativa* extract to copper oxide solution, the solution became black gray in color **(Fig.10.B)** . The color change confirms that the copper oxide was reduced and transformed into copper nanoparticles.

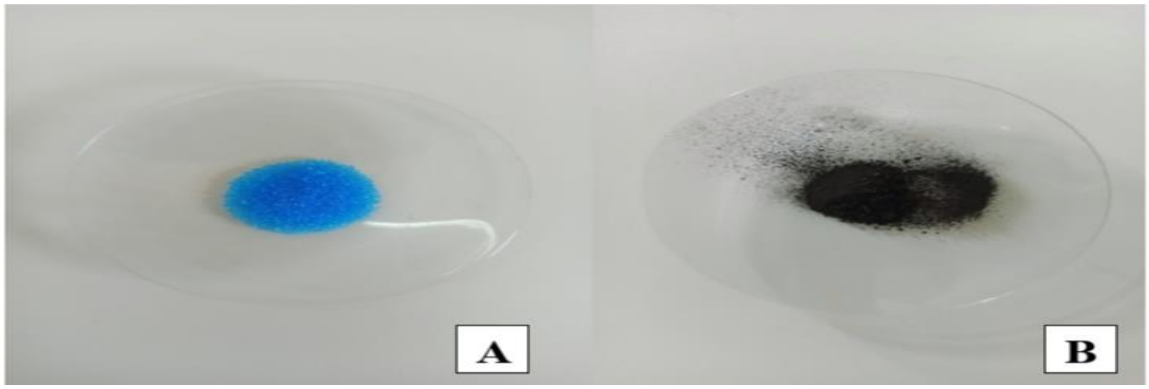

Figure 10: Biosynthesis of copper Nanoparticles (CuNPs) using aqueous extract of *M. Sativa*

1.2.2. UV-Vis spectral studies

The presence of nanoparticles was confirmed by obtaining a spectrum in the visible range of 200 nm-700 nm using UV-visible spectrophotometer **(Fig. 11).** From this analysis, absorbance peak was found at around 300, which was specific for Cu nanoparticles. Based on the UV-ViS spectra, the sharpness of the absorption peak was found to be dependent concentration ratio of *Medicago sativa*, thus, it was sharper with a higher concentration ratio**.**

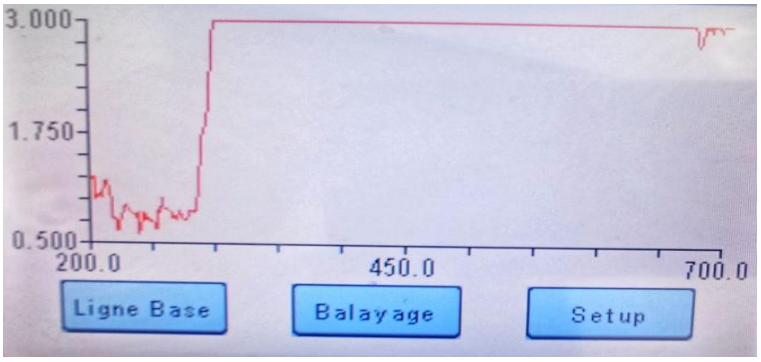

Figure 11: UV-Vis spectra of copper nanoparticles using aqueous extract of *M. Sativa*

1.2.3. SEM and EDX studies

SEM technique was employed to visualize the size and shape of copper nanoparticles. In **Figure**.**12**, SEM images were obtained with 10% of *M. sativa.* The SEM (JEOL MODEL 6390) used SEM grids which were prepared by placing a small amount of sample powder on a copper coated grid and drying under lamp. The formation of copper nanoparticles as well as their morphological dimensions in the SEM study the shape was oval and homogeneous with inter-particle distance. The shapes of the copper nanoparticles proved to be Multifaceted.

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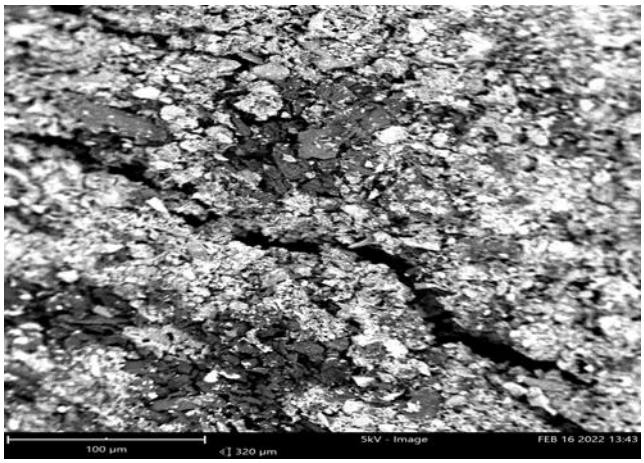

Figure 12: SEM image of copper nanoparticles formed by *M. Sativa*

1.2.4. XRD studies

A detailed analysis of the X-ray diffraction diffractograms showed that the main components of test powder were CuO nanoparticles as shown in **figure 13**.The XRD analysis of CuO demonstrated the peaks at 36.81°, 39.52°, 42.31°, 49.09°, 53.98°, 63.17°, 68.97°and 75.45°. The results of XRD analysis are consistent with the Inorganic Cristal Structure Database, ICSD 01-089-5898 for copper oxide.

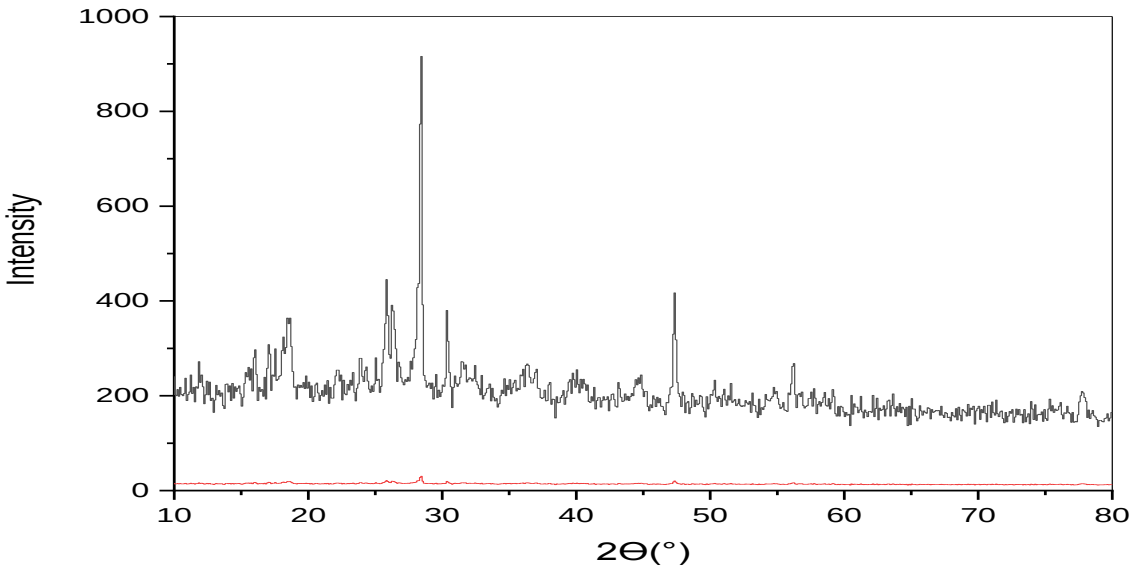

Figure 13: XRD pattern of the synthesized CuNPs using *M. sativa* extract.

1.2.5. FTIR spectroscopy

It has been shown that phytochemical analysis of *M. sativa* extract reveals whether the aqueous extract contains carbohydrates, glycosides and flavonoids. The presence of carbohydrates, glycosides and flavonoïds in *M. sativa* extract may play an important role in Cu reduction reaction. FTIR spectroscopy was used to characterize and identify the chemical composition of the CuNPs surface. As can be seen in **Fig.14**, the peak at 3400 cm-1 revealed that water and OeH absorption frequency.

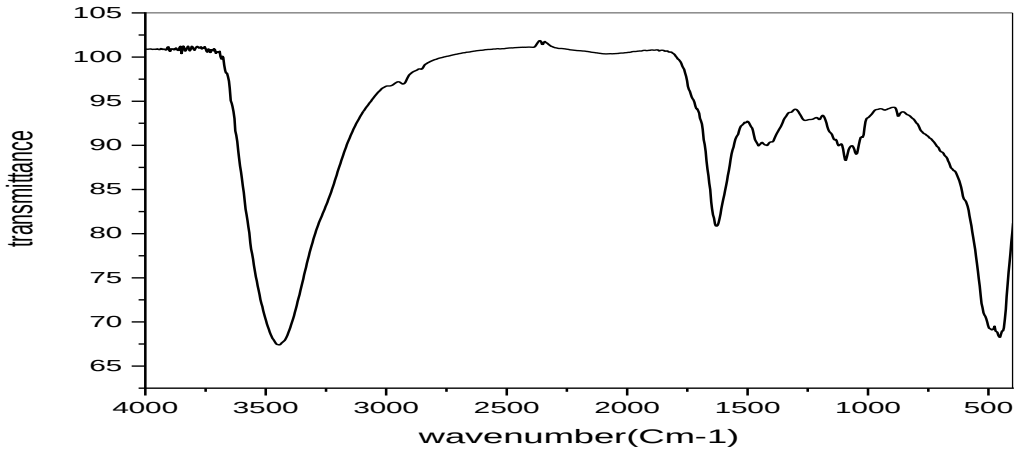

Figure 14: FTIR spectra of Copper nanoparticles using *Medicago sativa* extract

1.3. In vivo study essays of CuNPs and *M. sativa*

1.3.1. Growth parameters:

Result show a high signification ($p<0.01$) of weight gain in phenylhydrazine but show there is increase in relative liver, spleen and kidneys weight compared to control group. In CuNPs group, results showed a significant increase ($p<0.01$) in weight gain, with a decrease in the relative weight of the liver, spleen and kidneys. Treatment with *M. sativa* increase the weight gain with there is a decrease in relative weight of the liver and kidneys and there is no effect on the relative weight of spleen compared to the PHZ group **(table04)**.

Table 05: Body weight and organ relative weight in control and experimental rats

	Control (n=5)	**PHZ (n=5)**	**PHZ +CuNPs (n=5)**	**PHZ + *M. sativa* (n=5)**
Initial weight (g)	158.60 ± 4.25	152 ± 4.51	150.40 ± 3.37	151.80 ± 3.30
Weight gain (g/day/rats)	59.7 ± 29.1	11.11 ± 1.39**	80.0 ± 20.6NS NS	35.83 ± 8.99 * a
Relative liver weight (%/g tissue)	2.512± 0.059	2.658 ± 0.07NS	2.598 ± 0.09NS NS	2.606 ±0.07NS NS
Relative spleen weight (%/g tissue)	0.210 ± 0.002	0.211 ± 0.0006NS	0.1970 ± 0.003**b	0.217 ± 0.0089NS NS
Relative kidneys weight (%/g tissue)	0.255 ± 0.004	0.337± 0.06*	0.259 ± 0.01NS b	0.270± 0.006NS 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.

1.3.2. Hematological parameters

Hematological parameters illustrated in **table 5** show that, there is increase in WBC, LYM, PLT and MCV levels with a decrease in paremeters in HGB, HCT, GR and GRAN levels in hole the experimental rats in phenylhydrazine groups compared to the control.

Result show that there is a decrease of CuNPs in WBC, LYM, PLT, MCV with a small increase in RBC and HGB compared to PHZ group, in treatement of *M. sativa* show a high significant change ($p<0.05$ and $p<0.001$) in WBC,LYM, PLT and MCV but in RBC and HGB there is a small increase compared to phenylhydrazine group.

Table 06: Hematological markers levels of control and experimental groups control.

Parameter	Control (n=5)	PHZ (n=5)	PHZ +CuNPs (n=5)	PHZ + *M. sativa* (n=5)
WBC (10^9/L)	6.93±0.076	9.77± 0.73*	9.01±.070$^{* \text{ NS}}$	8.5±0.115$^{\text{NS a}}$
LYM (10^9/L)	4± 0.6	7.16±0.1003 **	6.5±0.5^{**a}	6.7±0.5$^{** \text{ NS}}$
GRAN(10^9/L)	2.3± 0.55	1.86± 0.13**	2.15± 0.35$^{\text{NS NS}}$	2.45±0.25 $^{\text{NS a}}$
HGB (g/l)	155 ± 0.151	149,75 ± 5,91 *	152±4,24$^{\text{NS NS}}$	135,40±0.500^{***b}
HCT (%)	42,61 ± 0.146	40,63 ± 0.181*	40.93±0.116$^{***\text{NS}}$	37,14±0.143$^{***\text{NS}}$
RBC (10^{12}/L)	8,37±0.29	7,75±0.260*	7.96±0.306$^{** \text{ NS}}$	7,92±0.287 $^{\text{a NS}}$
MCV (/L)	51 ± 0.420	53.3 ± 0.642**	52.575±0.669 $^{* \text{ a}}$	50.620±0.473 $^{\text{NS}}$ c
PLT (10^{12}/L)	786 ±0.557	813±0.249 *	772±0.390$^{\text{NS a}}$	733±0.543 $^{\text{NS NS}}$

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.

1.3.3.Biochemical parameters

Biochemical parameters are illustrated in **table06** which show that, there is a significantly increase of serum urea, creatinine and total bilirubin but a significant decrease ($p<0.001$) of ferritin and vitamin B12 in phenylhydrazine group, compared to control. Treatment by CuNPs and *M. sativa* ameliorate the most of previous parameters especially the CuNPs group compared to phenylhydrazine group.

Table 07: Biochemical markers level in the control and experimental groups

parameters	Control (n=5)	PHZ (n=5)	PHZ +CuNPs (n=5)	PHZ + *M. sativa* (n=5)
Creatinine (mg/l)	9.363 ±0.480	10.95 ± 0.50^{NS}	8.844 ± 0.48$^{** c}$	7.792± 0.657$^{** c}$
Urea (g/l)	1.113±0.196	1.753± 0.007***	1.136 ± 0.159$^{NS\ b}$	1.190 ± 0.144$^{NS\ b}$
Bilirubin total (g/l)	25.53 ± 0.00	34.83 ± 2.41**	30.65 ±1.21 ^{**a}	30.94 ± 4.97 $^{NS\ NS}$
Ferritin(ng/ml)	4.14 ± 0.963	1.927 ± 0.546*	2.223 ± 0.028$^{*** c}$	2.067 ± 0.268^{**NS}
Vit B12 (pg/ml)	3696±3276	84.48 ± 7.34***	104.63 ±6.28 ^{***a}	88.6 ± 14.7$^{** *NS}$

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.

1.3.4. Oxidative stress parameters

1.3.4.1. Malondialdehyde (MDA) and Super Oxide Dismutase (SOD)

Results of tissues MDA levels are presented in **table07** The levels of MDA in the liver, kidneys and spleen were significantly increased in the phenylhydrazine group compared to control. While tissular MDA levels were significantly decreased in the CuNPs group, as for the group treated with *M. sativa* showed a decreasing of the Tissular MDA levels Compared to phenylhydrazine group.

Results of liver, spleen and kidney SOD activities are presented in figure.... the levels of SOD in the liver, kidneys and spleen were significantly decreased in the phenylhydrazine group compared to control. Treatment with CuNPs and *M. sativa* shows an increase in SOD activities of all tisuues compared to the phenylhydrazine group **(table07)**.

Table 08: MDA and SOD levels in control and experimental groups

Parameters		Control (n=5)	PHZ (n=5)	PHZ +CuNPs (n=5)	PHZ + *M. sativa* (n=5)
MDA (nmol/g tissus)	**Liver**	9.75 ± 1.38	21.43±3.21*	5.882±0.252***C	24.29±2.25 * NS
	spleen	7.288 ± 0.586	14.95±1.17**	8.113±0.609NSC	8.510+0.61 NSC
	Kidneys	15.36 ±1.46	39.3±11.6NS	14.89±1.83NSC	17.04±1.53 NSC
SOD (UI/g tissus)	**Liver**	1.634 ± 0.067	1.2636±0.024***	1.7876±0.04**C	1.3438±0.05**NS
	spleen	1.725±0.037	1.3594±0.060**	1.6111±0.065 NSb	1.47±0.030***a
	kidneys	1.760±0.04	1.2614±0.055***	1.370±0.09 **NS	1.330±0.01*** b

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.

1.4. Histological results

As show in **(figure15)**, result of bone histological study indicated normal cells layer structure with space of the trabecular bone is large and bone marrow disconnected in control group, conversely in phenylhydrazine group the histological results show a total damage with modification at the structural level of the bone there is a highly vascularized space stenosis of trabecular bone and bone marrow connected due to broken bone cells (filled with red blood cells), in **figure(15)**. Histological observations of the bone of the rats treated with *M. sativa* extract show a partial correction in bone marrow compared to phenyl hydrazine . Finely, Cu NPs tied to *M. sativa* group the histological result show normal morphological of cells layer with few a bone lesions compared to phenylhydrazine group **(figure15)**.

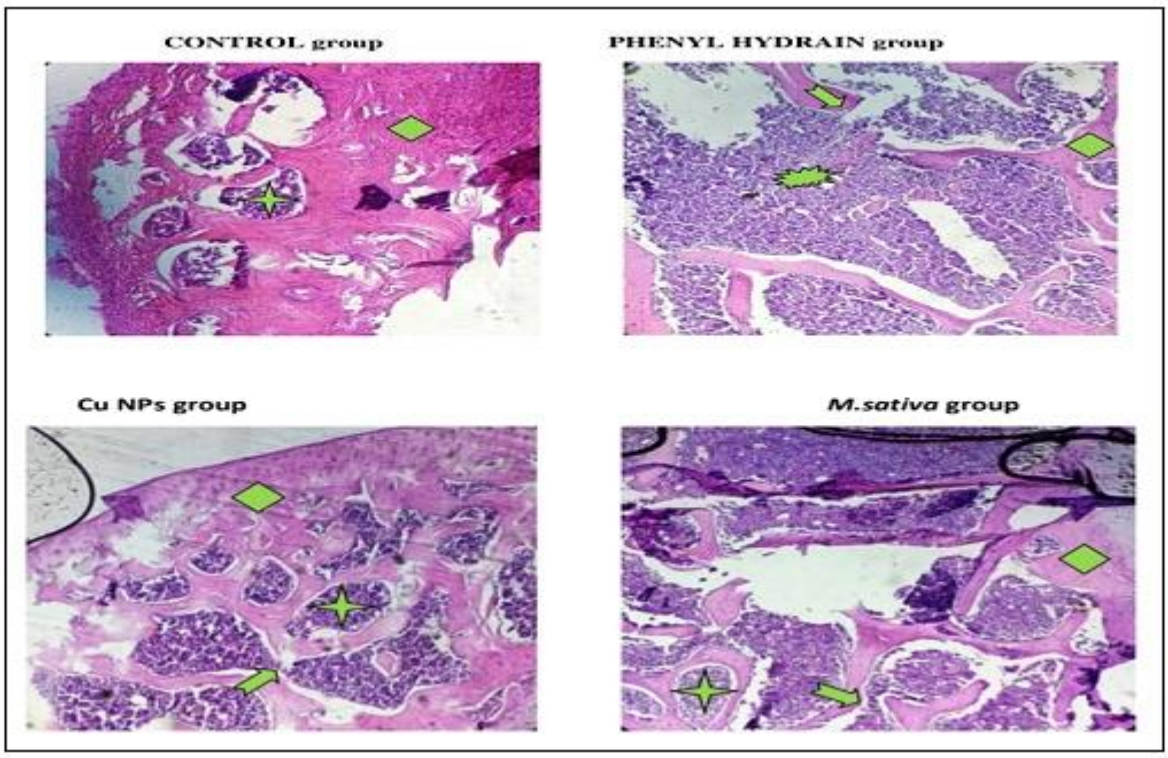

Figure 15: Bone Histological examination (×400)

= bone marrow disconnected = trabecular bone , = bone marrow connected (fracture at the bone), = highly vascularzed.

For kidneys histological study as show in **(figure16.)** results indicated normal cells layer structure glomerulus similar sizes and narrow bowmen's space, conversely in phenyl hydrazine group the histological results show a bowmen's space expansion with hemorrhagic necrosis and inflammation at the level of tissue cells. Histological observations of the kidneys morphology of the rats treated with *M. sativa* extract show slight correction in morphological with the survival of necrosis and the expansion of a bowmen's space at the level of some cells .

Finely in CuNPs using *Medicago sativa* extract group the histological result show almost completely a correction in morphological better than a *M. sativa* group, with the appearance of bleeding at the level of the cells layer.

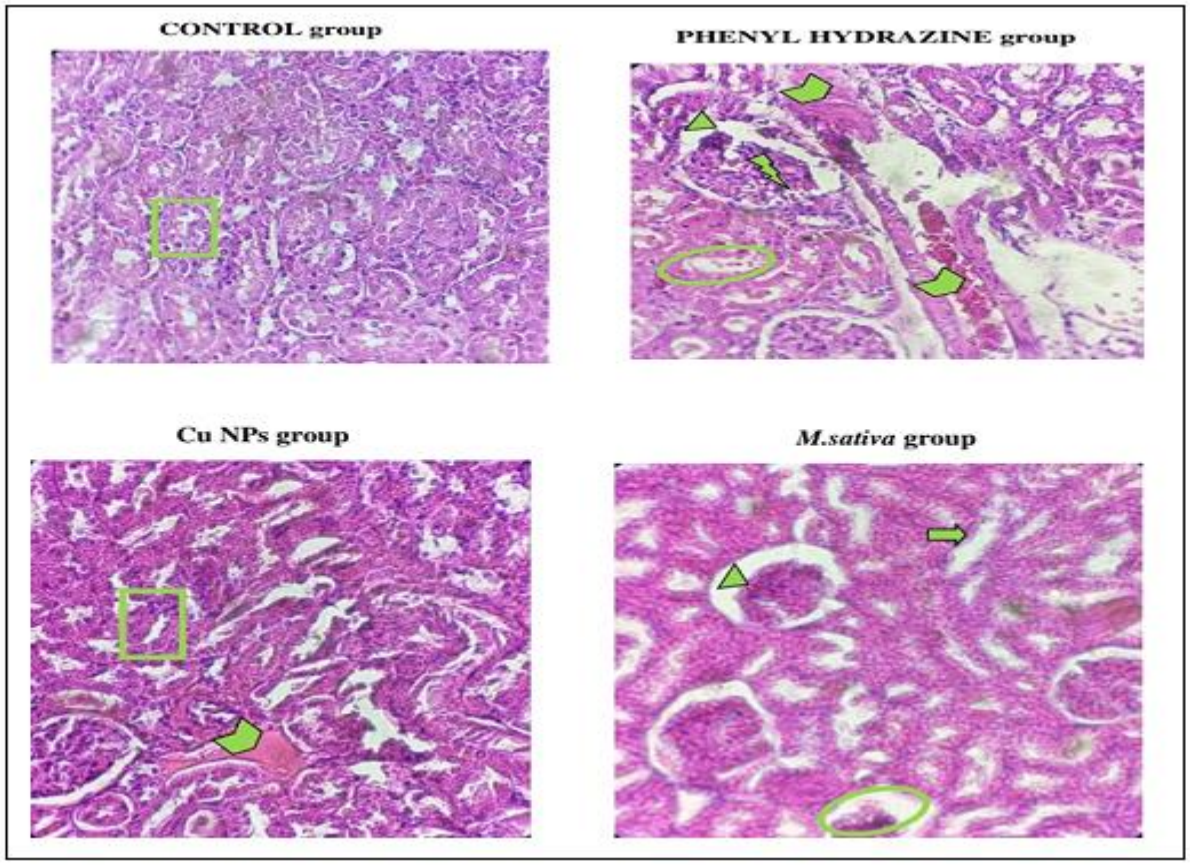

Figure16: Kidneys histological examination (×400)

= Glomerulus, = Bowmen's space expansion, = Bleeding (hemorrage), = Inflammation, = necrosis, = tubule runal.

For spleen as show in **(figure17)**. In control group results indicated normal morphology structure of white bulb, red bulb where they both have the opposite side. Compared to phenylhydrazine group **(figure 17),** show a necrosis with the high number of white blood cells and their miscing with red blood cells, and this is evidence of the presence of inflammation at the level of the cell layer. In group treated with *M. sativa* extract **(figure17)**,the histological results show a partial correction in morphological ,and there are some parts that remained on the effect of phenyl hydrazine where it shows the appearance of necrosis. In CuNPs group **(figure17)**,the histological results show almost total correction in morphological and there are also a some destroyed necrosis by phenyl compared to phenylhydrazine group.

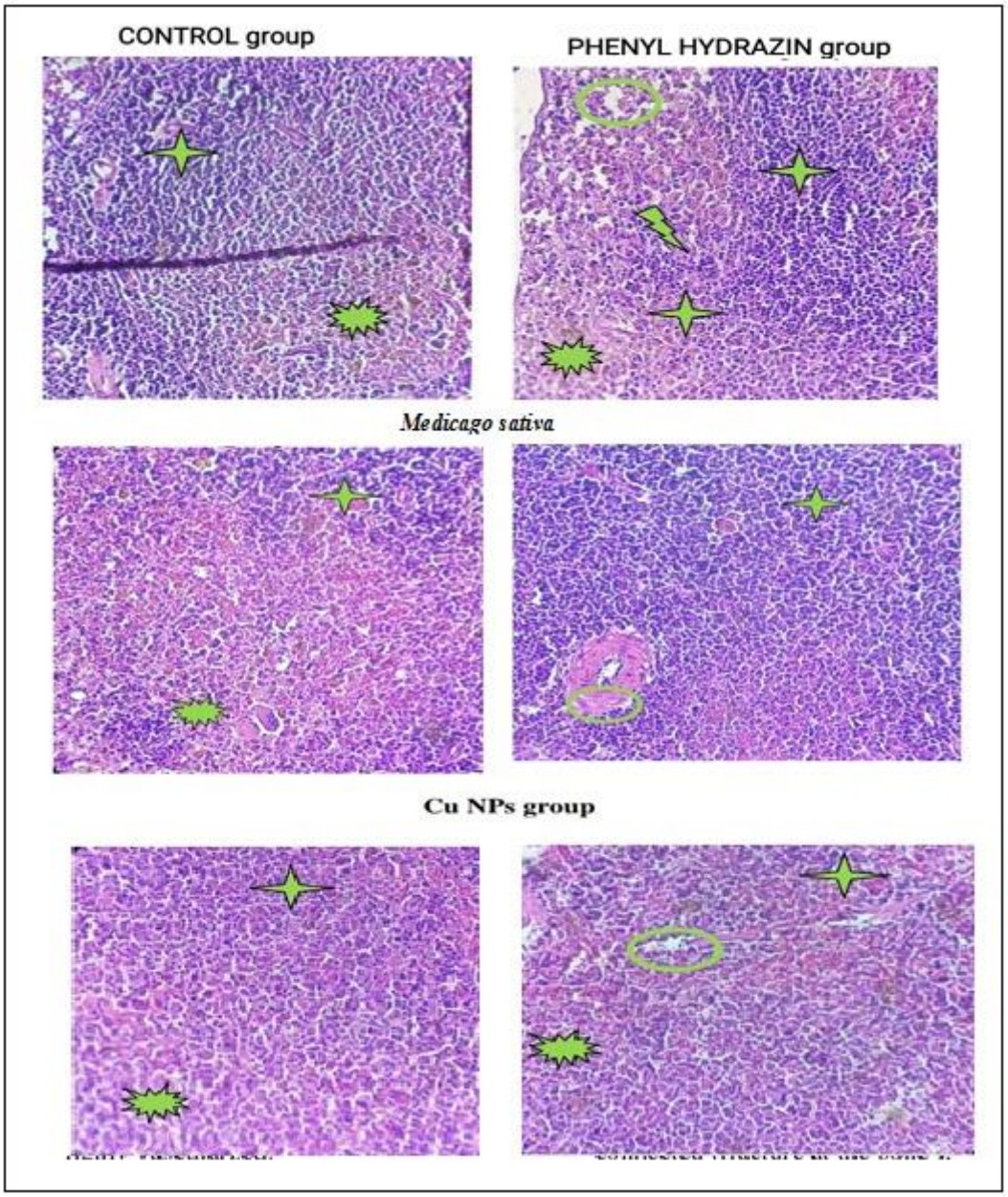

Figure17: Spleen histological examination (×400)

= red bulb, = white bulb, = necrosis, = inflammation.

2. Discussion

The objective of our study is green preparation of CuNPs using *Medicago sativa* and evaluation of their effect against experimental anemia induced by phenylhydrazine in Wistar rats.

2.1. In vitro assay of *M. sativa*

The results of phytochemical essays show that aqueous extract of *M. sativa* with different important second metabolic (flavonoids, phenols, carbohydrates, saponoside, and terpenoids) but absence tannins alkaloids. According to (Bora & Sharma, 2011) phytochemical result same of our result. Secondary plant metabolites have biological properties such as antioxidant activity, antimicrobial effect, antidiabetic effect, anti-inflammatory effect, hypolipidemic effect, anxiolytic effect, dermatological effect, immunological effect, cytotoxic effect (Ali Esmail *et al*., 2021). As appeared in (Eugeniuses, 2014) show Much attention is given to effects of saponins occurring in alfalfa. The studies on animals (macaques) did not demonstrate any toxic effect of alfalfa saponins. Saponins have been implicated in the total cholesterol level reduction and in the enhanced secretion of bile, gastric, pancreatic and intestinal juices. The studies revealed that depending on their chemical structure, they may display antimycotic and antibacterial actions-mainly against selected yeast pathogenic to man. Therefore, they may also have immune stimulatory effects. The highest activity of alfalfa spawning was observed against Gram-positive bacteria, e.g., *Staphylococcus* eraser *Enterococcus faecalis*. The in-vitro studies demonstrated their efficacy as anticancer agents and confirmed their capability to inhibit the development of leukemia cell line in human. The total phenolic and flavonoids compounds content in the aqueous *M. Sativa* extract are higher than values reported by (Karimi, *et al*., 2013) which suggests that the concentration is dependent on the aqueous extraction method and characteristics of the sample.

The HPLC result revealed the presence of phenolic, p. Coumaric acid and flavonoids, compounds such as naringin and this is similar to the result of another study In addition to these vehicles, he directed several other vehicles (Karimi *et al*., 2013) (Hcnif, 2015).

The anti-oxidant activity result studies have reported that *M. Sativa* plant extract carries a strong antioxidant effect Because they contain many phenolic, flavonoids and saponins all show high potential for antioxidants . They are considered to be powerful free radical sweepers as described in FRAP tests by reducing iron by donating hydrogen, this is proportionate to the extract's concentration. Free radicals are known to play a quiet role in the automatic oxidation of unsaturated fat in nutrients and phenolic, flavonoids and saponins fight free radicals. Since our

extract is rich in these compounds, it has contributed to antioxidant activity. This is similar to several studies, including (Bora&Sharma, 2011;Wrona & Górecki, 2016; Wangm, 2021).

2.2. Biosynthesis and characterization of copper nanoparticles using *M.sativa*

According to our study, green synthesis of CuNPs from *M. sativa* extract and the addition of CuSO4 Caused the mixture to change color from green to black We think this indicates the formation of CuO And that's similar to a study(Ouidad *et al*., 2020) ,And that's why our plant extract is rich in phenolic compounds, flavonoids, saponins ,carbohydrate and nargenin that have antioxidant properties and biological reductivity in the formation of nanoparticles. It reduces copper ion status to copper condition and simultaneously oxidizes it to form nanoparticles, we suggest that hydrogen ions are divorced during net diversion. For the enol form of flavonoids to the quito shape Reducing copper ions and the formation of CuNPs nanoparticles and so compared to the following studies (Mroczek-sosnowska *et al*., 2013;Wangm, 2021**)**.

UV-Vis spectroscopy is one of the essential systems used to determine the primary existence of metal nanoparticles in a liquid medium. The color change demonstrating the presence of cu nanoparticles was further characterized by UV–Vis spectrophotometer and observed by taking readings at a distinctive temperature. The absorption peaks show the UVVis spectra of copper nanoparticle formation at temperature room using *Medicago sativa* nanoparticle extract. The peaks shows due to surface plasma resonance of copper colloid for diverse temperature were seen in the range of 200 to 700 nm spectral lines were observed and the UV visible spectra. The intense SPR bands were 300 observed around ~435 nm. Our results confirm the observations that the copper nanoparticles are formed.

About FTIR spectra of CuNPs, results demonstrated the peaks at 3500, 1600 and. According to; The band at 3452 cm−1 corresponds to—polymeric‖OH stretching mode. The absorption at 1629 cm −1 represents amide and Open chain amino (\\C_N\\). 1388 cm−1 relates methyl C\\H says. /Siam bend. This confirmation proposes that the protein particles could perform the capacity of the arrangement and adjustment of CuNPs in the aqueous medium (Popa *et al*., 2022).

2.2.1.XRD analysis

X-ray diffraction (XRD) analysis showed the presence of a large number of sharply pronounced reflections, which are indicative of the crystallinity of the synthesized particles structures (Shutov *et al*., 2017). The ecofriendly method of nanoparticles synthesis is confirmed by XRD technique as showing in study of (Elumalai & Velmurugan, 2015) which used *Azadirachta indica* (L.) for synthesizing CuO nanoparticles.

The SEM image showed relatively spherical shape nanoparticle formed with diameters in the range of 35-55 nm. A similar phenomenon was reported by (Chandran *et al*., 2006) and (Udayasoorian *et al*., 2011). Energy dispersive spectrometry (EDS) micro-analysis is performed by measuring the energy and intensity distribution of X ray signals generated by a focused electron beam on a specimen.

2.3.In-vivo study

2.3.1. Growth parameter

While doing our work, we periodically tracked the weight of the rats, and our results were as follows, as the average weight in the PHZ-treated group was lower compared to the control group, and there was a slight increase in the mean weight in both *M. Sativa* and CuNPs treated groups compared to the PHZ group, and we explain the weight loss in the PHZ due to the toxins caused by phenylhydrazine in the body and a defect in all functions and body, as well as anemia, which in turn leads to loss of appetite and thus weight loss, and this was confirmed by the following study, where IDA patients suffer from decreased appetite and paradoxically increased activity of the hormone ghrelin compared to healthy controls. IDA therapy enhances appetite and lowers ghrelin levels. Future studies are needed to explore the mechanism of this paradoxical ghrelin activity (Ghrayeb *et al*., 2020) as well as explain the increase in weight in both groups treated with *M. sativa* In this study, ingestion of alfalfa extract was associated with improved animal weight. It appears that the potential effect of alfalfa by decreasing metabolic energy and increasing appetite could improve body weight in rats (Raeeszadeh *et al*., 2021).

2.3.2.Hematology and biochemical parameter

The result WBC, LYM and GRAN show an increase in phenylhydrazine group compared to the control .(Berger, 2007) PHZ injected at a dose 90mg/Kg rats causes a leucocytosis with neutrophilia and lymphocytosis at its maximum. It was found to be mutagenic and lymphoid cell

activator (Dornfest *et al*., 1990). PHZ-induced anemia is also responsible for immune activation. In this respect, PHZ can cross red blood cells and binds with circulating autologous antibodies. This antigen-antibody complex is receptors which triggers phagocytosis in the spleen and liver (Pandey *et al*., 2014).

In the treatment of *M. Sativa* group and the CuNPs group note improvement in value WBC, LYM and GRAN. This is due to the fact that *M. sativa* has an anti-inflammatory effect. According to (Hong, 2009) supplements with *Medicago sativa* ethyl extract have discouraged the production of proinflamatory cytokines and mitigated acute inflammatory risks in mice. The extract significantly reduced the production of IL-6 IL-18 and activation activity of NF Kappa B for metogen-stimulated RAW264.7 cells. Furthermore, the extract showed much lower beta levels than TNF-a serum, IL-6 and IL-1 at 9 hours after the LPS challenge, and much higher survival rates than the control group. Also The link between Cu and innate immune function has been recognized for. recorded that mild Cu deficiency in humans and animals was often characterized by neutropenia (Mckie *et al*., 2000;Donovan et al., 2000). This condition was partly associated with a decrease in the number of neutrophils in circulation, and thus a role for Cu in differentiation of leukocytes, maturation and proliferation was proposed (Vogel, 1984).

Decrease in red blood cells (RBCs) and hemoglobin (HGB), followed by lack of frrition and vitamin B12 in phenylhydrazine group compared to the control. (Jiang *et al*., 2016) PHZ was peritoneally administrated into mice at different doses a significant reduction of RBC count and HGB concentration were observed in PHZ-treated mice. (Masaratana *et al*., 2012) phenylhydrazine injection in Hamp mice caused a significant change in H-ferritin and TfR protein by reducing them.

We explain the change in red blood cells (RBCs) and hemoglobin (HGB) as a decrease in that PHZ group it causes oxidative stress in RBCs and generates reactive oxygen species (ROS) in the RBCs this ROS reacts with haemoglobin and changes the oxyhaemoglobin in to methaemoplobin. PHZ induces a reactive oxygen species formation which results in Peroxidation in lipid and oxidative degradation of spectrin in the membrane Skelton. After that PHZ translocates the phosphatidylserine from inner to outer of the plasma membrane and causes the membrane lipid peroxidation due to lipid peroxidation RBCs enter in the spleen and uptake by the macrophages. It is a signal for Phagocytosis of cells under programmed death by macrophages (Pandey *et al*., 2014). Phenylhydrazine is absorbed through all pathways; some binds to the hemoglobin of red blood cells. It is completely transformed into several metabolites After absorption, a part quickly enters the red blood cells where it reacts with oxyhemoglobin to form methemoglobin,

phenylhydrazine, phenyl radicals and reactive oxygen species that cause cell membrane damage, when methemoglobin is formed, it leads to liver, spleen and kidneys damage (Bonnard *et al*., 2007). This confirms our experience in tissue death of studying organ cells caused by PHZ. We discuss the deficiency of ferritin protein (an iron storage protein) in the phenylhydrazine group, that PHZ effect on the formation of red blood cells in bone marrow. In another experiment, similar to ours, it indicates that the hematopoietic system of bone marrow was remarkably activated under hemolytic stimulation. By contract, consistent with reduced RBC content (Jiang *et al*., 2016).

We advertise the improvement of red blood cells, hemoglobin and a very great improvement in the tow ferritin in a CuNPs group that it can help the formation of red blood cells. Where the (Environmental, 2013) shows it, effects of copper deficiency can include anemia, This also shows its effect on the enzyme feroxidase I and feroxidase II have the ability to oxidize iron (Fe 2+) to iron (Fe 3+), which are connected to protein transferring for transportation to the red blood cells and blood formation. Although feroxidase activity of these two copper enzymes is still not thoroughly understood, the physiological significante and the involvement of copper in iron metabolism has been clearly demonstrated(Angelova *et al*., 2011). In the group treated with *M. sativa* an increase in the value of vitamin B12 was observed. Used to lift concentrate over an alternative to iron and folic acid supplements. The result revealed that the leaf concentrate is effective, and more palatable, alternative to Fe and folic acid supplements for treating anemia (Kundun singh Bora & Anupam Sharma, 2011).

Hemolysis interferes with accurate determination of bilirubin; nonetheless increased levels of bilirubin in the rat. After PHZ injection, increases in bilirubin have been observed, with kinetics similar to hemoglobin(Pandey *et al*., 2014). Bilirubin is a product of heme catabolism. Phenylhdrazine (PHZ) is a strong oxidant agent and potent hemolytic known to cause anemia. PHZ-mediated hemolysis induces hepatic hemeoxygenase, which, in turn, causes hyerbilirubinemia (Boizot *et al*., 2006).

The level of bilirubin total decreased in the group treated with *M. sativa* , because this plant contains quercetin (one of the most common flavenoids) that reduces the level of total bilirubin compared to oxidative stress induced by PHZ in rats (EL-sayed *et al*., 2021).

Through the results of our study on the percentage of urea and creatinine in the PHZ-treated group, we notice an increase in the percentage compared to the control group, and we notice a significant decrease in the *M. sativa* The active treated group compared to the PHZ-treated group. We also note that CuNPs-treated group preserved, but this decrease was less than in sativa group.

We explain the high percentage in the PHZ group that when the kidneys ability to eliminate wastes decreases due to abnormalities in the kidneys caused by phenylhydrazine, the amount of urea and creatinine in the blood increases. And biochemistry, as well as oxidation (Pandey *et al*., 2014), and we explain the decrease in the percentage in the group treated with *M. sativa* and CuNPs as that the repairs that took place at the level of the kidneys led to an improvement in the ability of the kidneys to eliminate wastes in the urine, so the percentage in the blood decreases, and this is proven by the following study. It is possible Because of its excellent ability to clear compounds from the kidneys and increase blood flow to the kidneys, especially areas containing the amino acid cysteine, plasma levels of urea, creatinine, and uric acid are the main indicators of kidneys function (Vogel, 1984).

2.3.3.Oxidative stress

The chemicals are known to cause oxidative stress in red blood cells. The Toxic kinetics of phenylhydrazine for rapid absorption have been studied, regardless of the route of administration., hydrogen peroxide has been identified as major receptor. (Rationale *et al*., 2020) Phenulhydrazine may damage the liver, spleen and kidneys. High and repeated exposure can damage blood cells, causing anemia (low blood cells count) (Standard, 2004).

In the current study, the role of the *M. sativa* extract and CuNPs on the toxicity of phenylhydrazine was investigated in female rat. The results of our experiment showed that the phenylhydrazine treatment group increased the MDA concentration in the kidneys and spleen tissues compared to the control, with a decrease in the SOD activity in the three organs compared to the control, which indicates an increase in lipid peroxidation. MDA is an end product of lipid peroxidation compared to control group, and this may be considered a late biomarker of oxidative stress and cellular damage (Derouiche *et al*., 2018). (YEW KOON, 2000) as studied in the following trial assessed lipid peroxidation in kidneys tissue by determination of levels of MDA end products. The kidneys of rats treated with phenylhydrazine were higher than the kidneys of control rats. During induced hemolysis, MDA was significantly increased in phenylhydrazine (Elaby *et al*., 2018). In a previous experiment in mice injected with phenylhydrazine, a significant increase in MDA and H2O2 in the liver, spleen and kidneys was observed in mice serum, and PHZ injection in mice was associated with a significant increase in serum MDA and plasma H2O2 levels (Djoko *et al*., 2015).

Discussion in the cellular membranes observed in this study agreed upon. The level of MDA are increased in liver and spleen, fat products was expected, after treatment of PHZ, because the

liver is the main storage location of the iron and the phase is anxious increases in our liver and spleen. Peroxidation fat caused by iron is a well thoughtful phenomenon. In addition, the reduction of membrane in the hepatic microscopy because of the PHZ, as shown in these studies, agree with damage caused by the examination not only for fat but also for proteins and with fat caused by iron and protein damage. In fact, changes in the membrane structure because of both examination(Masartana *et al*.,2012) and the iron was observed wrongly. A growing amount of fat peroxide products has also been found after PHZ treatment in blood plasma. These products are derived from solid tissue, when the cellular membranes were exposed to PHZ and / or iron (Djoko *et al*., 2015).

According to (Hill and ThorAlly 1982, Clemens *et al*., 1984; Amer *et al*., 2004) PHZ increase reactive oxygen species (ROS) and decreases glutathione (GSH); these effects are reversed by N-acetyl cysteine, a known ROS scavenger (McMillan *et al*., 2005). PHZ associate primarily with the plasma membrane and cause a perturbation of structure which leads to enhanced permeability . they penetrate intracellular space . Thus, its administration in vivo may lead to the production and accumulation of hydrogen peroxide in amounts above the detoxifying capacity of cellular protective mechanisms . Additionally, phenylhydrazine has been shown to generate superoxide, hydroxyl radicals, and phenyl radicals . These radicals have all been implicated in its cytotoxicity. (Angeles, 1980) PHZ induces a reactive oxygen species formation which results in peroxidation of lipid and oxidative degradation of spectrin in the memberane sklton (Pandey *et al*., 2014). On the other hand, MDA, is more cytotoxic to cells affects the membrane structure and causes further destruction of the cells, accompanied by hemorrhagic necrosis (Mehmet Ibrahim Turan *et al*., 2013).

Interestingly, when endogenous antioxidant systems are depleted to combat cellular damage from oxidative stress, we may have to rely on exogenous antioxidants(Anbara *et al*., 2018). In recent years, there has been a growing interest in natural antioxidants. The literature recognizes that the replacement of synthetic antioxidants by natural antioxidant can have several advantages and much of the research on natural antioxidants focused on phenolic compounds, in particular flavonoids as potential sources of natural antioxidants (Dontha *et al*., 2016). In this context, secondary metabolites of plant origin, such as flavonoids, phenolic compounds and steroids are well known for their antioxidant efficacy, and saponins is one of the plant steroids that is well known for its various therapeutic benefits which are mainly mediated through the antioxidant mechanism (Anbara *et al*., 2018). The richness of our plant like saponins makes it one of the most powerful antioxidant plants. According to (Viswanatha *et al*., 2017), Saponins supplementation

(100 mg/kg) can attenuate the antioxidants by increasing the level (sod..), and decreasing levels of MDA, TNF-a, IL-1b Exploring the mechanism of alfalfa saponins in the regulation of oxidative stress. In general, the content of free radicals maintains a dynamic homeostasis in cells. Once this balance is destroyed, it will cause oxidative damage to cells, leading to redox-related substrates content changes. T-AOC can be used to indicate the total antioxidant capacity of organisms. MDA is one type of lipid peroxidation metabolite that is produced through the non-enzymatic system when oxygen free radicals attack the membrane of polyunsaturated fatty acid (PUFA). LDH It is a stable cellular enzyme that can be rapidly released into the extracellular plasm once the cell membrane is damaged. the amounts of MDA indirectly reflect the degree of cellular damage. Currently, researchers have proven that most saponins have a certain antioxidant capacity. Furthermore, MDA contents were significantly lower in the oxidative stress model pre-incubated with alfalfa saponin, indicating that alfalfa sponin can increase antioxidant enzyme activity and enhance the ability to scavenge free radicals (Cui *et al*., 2020).

The results of the experimental rate treated with *M. sativa* extract and CuNPs showed that treatment with the *M. Sativa* aqueous extract and CuNPs after disease with phenylhydrazine improved the antioxidant system, in which the level of MDA decreased and SOD activity increased. This confirms the results of the antioxidant test in the plant, as it shows that it has a significant antioxidant effect.

Medicogo sativa is one of the most popular medicinal plants traditionally used to treat kidneys pain and antioxidants (Kluwer, 2005; Herbal, 2008) the chemical report of the *M. sativa* plant revealed that it contains flavenoids and polyphenols, which makes it an antioxidant. Where it can reduce MDA and increases the SOD by polyphenols and flavonoids containing a hydroxyl group in its structure, making it a very important antioxidant activity. Hydroxyl groups in this nucleus donate hydrogen and electron to hydroxyl, peroxyl and peroxinterite roots, stabilizing them and producing relatively stable flavonoid roots (Reilly, 1989). The specificities of the sativa leaf extract were investigated. Overall results showed that garlic acid, perogalol, salicylic acid and caffeic acid such as phenol, naringnin, apigenin, kercetin, mercetin and dedzene are flavonoids and isoflavonoid main in the stevia leaf extract. The functional properties including antioxidants, inflammatory inhibition activities and XO observed in this study are attributable to the presence of phenols and flavonoids. Some flavonoids and phenolic compounds such as perogalol, galic acid, narngene and corsetin possess antioxidant properties as well as anti-inflammatory activities (Karimmi *et al*., 2013).

CuNPs can reduce MDA effete and In our study phenylhydrazine decrease the reduced superoxide dismutase (SOD) level. SOD is probably the most important antioxidant present in the cells. Therefore, Results obtained in our study also showed that the treatment CuNPs relieves the state of oxidative stress, it's an important metal in growth and evolution. And an important ingredient for many enzymes, especially Antioxidant enzymes: cytochrome, lysyl, ascorbic ketocholic, tyrosinase and superoxide dismutase (SODs) (Mroczek-so., 2013) It is a non-enzymatic antioxidant (Shen *et al*., 2021).

SODs constitute a very important antioxidant defense in the body Several studies have been performed that reveal the therapeutic potential and physiological importance of SOD The enzyme can serve as an anti-inflammatory agent and can also prevent precancerous cell changes. It is one of the most important antioxidant enzymes where everything is formed superoxide anion free radical (O2-) into molecular oxygen and hydrogen peroxide (H2O2) (Younus, 2018).

In our study in CuNPs-treated proximity Note retention in MDA ratio Offset by high SOD enzyme in both liver, spleen and kidneys The effect of CuNPs was greatest in the liver Compared to kidney and spleen And this is because the liver is the first copper metabolic pathway Hence, the CuNPs we have created has an antioxidant effect. This is identical to result (Shen *et al*., 2021).

2.3.4.Histological analysis

As shown in **(Fig15)**, the result of normal cells indicated a layer structure with an area of trabecular bone and large marrow and bone cut in the control group, on the contrary in the phenylhydrazine group the histological findings and total damage appear with modification at the structural level of the bone there is a stenosis The area of high blood vessels from the trabecular bone and bone marrow connected with a large area due to the broken bone cells filled with red blood cells, and this causes bleeding, which in turn leads to a decrease in the number of red blood cells that are not completely immature, which in turn leads to the anemia disease, as stated by the following experiment that we inferred on her Bleeding in the bone marrow occurred in animals that were given phenylhydrazine for more than 10 days, and perhaps due to the toxic effects of phenylhydrazine on the bone marrow. In the microscope it has been observed a discrepancy between the extension of the blood cells and the occurrence of endothelial network accumulated colloid It seems that this disparity to be added after stimulation of bone marrow. The formation of the walls in the capillaries of the newly formed obviously by fat cells with marrow and this hairs finally appeared as pockets bone marrow. Later, also of red blood cell-cell colonies of cells and a component of granulocyte it appeared, around the pockets (Säterborg, 1974).

PHZ plays role in electron transfer leading to the formation of free radicals. Stimulation of the bone marrow could also be induced by PHZ colloidal accumulates only within the sinusoids of the Bone marrow (Duckles *et al*., 1937).

The figure (15), according to the notes histological bones rat treated with the extract *M. sativa* partial correction in the bone marrow, compared to phenylhydrazine as the alfalfa plant began in the treatment of bone cells and re-built as well as by reducing the contact between the bone marrow and thus the beginning of reducing the density of red blood that causes cells, bleeding as the results appeared Kmalk in the stand next Alfalfa (*Medicago sativa*), dandelion (Taraxacum officinale) root or leaf, burdock (Arctium lappa), and yellowdock (Rumex crispus): have traditionally been used to fortify and cleanse the blood. For mild cases of anemia, they may help bring levels of hemoglobin into normal range (Oleszek *et al*., 1990)Accurately, Cu NPs were attached to the *M. sativa* group, and the histological result showed the normal shape of the cell layer with a few copper bone lesions, as the bone cells were restored and the bone marrow area became separated and naturally, and this led to the presence of a normal number Red blood cells this is due to the copper particles associated with the alfalfa, which worked to treat the source of red blood cells, which is the bone marrow. The etiology of anemia in copper deficiency is complex and multifactorial. Ceruloplasmin, a major copper-carrying protein in the blood, oxidizes iron to a ferrous form allowing iron to be transported in the circulation and bind to transferrin. Another important component in the interaction of copper with iron is Hephaestine, which is a copper-dependent peroxidase (Cohen *et al*., 1990).

The copper has proved a necessary assistant factor in the configuration of hemoglobin. This study was conducted to determine whether anemia is a lack of number blood cells with the awareness that copper is widely distributed in foods and that the quantity required to produce hemoglobin accurate, the medium diet of adults will likely be enough to provide copper enough to stimulate blood-component action (Choudhury, 2019).

For spleen as they appear in **(figure17)**, In the control group, the normal group results indicated in the white blood cells structure, the red blood cells where both are the other side. Compared to the phenylhydrazine Group **(Figure17),** the necrosis appears with the high number of white blood cells and this led to an inflated in the spleen size and mistake red blood cells, this is evidence of the inflammation at the level of cell layer and this is in the line with The following study where the PHZ effect on the spleen. PHZ immunosup is a role in the transfer of the electron leads to free radical configuration. The bone marrow formation of meta-haemoglobin and Heinz body formation are the opposite effects of PHZ toxicity. An increase in spleen weight due to the

excessive rush of iron results in the EPO activity of spleen, this condition is termed as splemegaly. PHZ induced anemia activates immune reaction, which triggers phagocytosis within the spleen and building of EPO receptors (Duckles *et al*., 1937).

M. Sativa extract **(form17),** histological results show a partial correction in the morphology, this indicates that the plant began to repair the place of phenylhydrazine by degrading it, and this is as stated in the following study, among a large number of drugs, 163 plants from 73 families were found. Effective in the treatment of diseases of the liver and spleen (Timbekova *et al*., 1996). Finally, in the CuNPs group, the histological results showed a near-complete morphological correction, and this is after adding copper, a study that works to take advantage of the iron reserve in the spleen, which in turn is the main component of hemoglobin, and this as it appeared in the following study that adding copper to A low-iron diet resulted in depletion of reserve iron in the spleen. When copper was added to a diet with adequate iron, the amount of spleen iron increased significantly, but iron alone did not lead to this increase. (Bhattarai *et al*., 2021) There are also some destroyed of phenylhydrazine compared with phenylhydrazine group possible any return to a period of treatment that was short-term.

results of kidneys histological study indicated that there are similar sizes of the normal cell layer structure and a narrow bowmen's space, and in contrast to the phenylhydrazine group, the histological results show an expansion in bowmen's space with hemorrhagic necrosis as a result of hemolysis caused by phenylhydrazine as shown in the following study increased oxidative damage in the kidneys.

Hemoglobin and its catabolic products, heme and iron, are known to catalyze several oxidative reactions that may damage tissues, and HP has been shown to mitigate hemoglobin-mediated oxidative damage, especially lipid peroxidation that begins with hemoglobin . In addition, Severe intravascular hemolysis or transfusion of hemoglobin solution often leads to acute renal failure and concomitant depletion of serum Hp12. Therefore, we hypothesized that the kidneys of PHZ rat would be particularly susceptible to oxidative damage, particularly during hemolysis (Lim *et al*., 2000) and inflammation at the tissue cell level.

Histological observations of the overall morphology of mice treated with *M. Sativa* extract showed a slight correction in shape, which indicates that the plant worked to repair damaged cells. Researchers believe that antioxidant compounds can play a role A protective against these diseases Alfalfa, *Medicago sativa* L, is one of the most famous transmission lines- medicinal plants are used to treat and prevent many Diseases. Alfalfa contains vitamins (A, B1, B6, B12, C, D, E and

K), amino acids, sugars, proteins, minerals (iron, zinc, Cu, Al, B, Cr, Co, Mn, Mo, Se, Si, Na, Ca, P, K, Mg), Other feeders (Lnamul, 2004) . Because of its richness in vitamins and phy-Toestrogens, this plant is used as a food additive in several Countries Phytochemical studies indicated that this plant Contains a variety of secondary metabolites including fonoids, alkaloids, phytoestrogens (comsterol, daidzein, Genistein, pyocyanin), and coumarin as an effective anti-Oxidants Now, the question is whether the offense- Aggregation of this plant extract can block the kidneys (Kumar *et al*., 2010). With the survival of necrosis and the expansion of bowmn's space at the level of some cells. This is because the treatment period was short Precisely in CuNPs using *Medicago sativa* extract group, the histological result shows almost better morphological correction than *M. Sativa* group, which indicates that nanoparticles associated with *M. Sativa* worked on the repair of kidney tissue and this was shown in the following study. Renal imaging and delivery of iron therapy with Ferumoxytol for patients with CKD or ESRD who lack adequate erythropoietin production. (Choyke & Koboyashi, 2006) With the appearance of bleeding at the level of the cell layer, and this is due to the fact that the treatment period was in a short period.

Conclusion

Conclusion

Anemia is a real health problem in daily medical practice. It is iron, folate and vitamin B12 deficiencies are the main causes of the onest of anemia. diagnosed in both young and old people. Biologically, macrocytic anemia has a vitamin deficiency or a problem associated with the morbidity of the subjects. At the etiological level the underlying causes of anemia are dominated by malnutrition, hemoglobinopathies and many infections associated with hygiene. These etiologies vary according to the age and sex of the patients. So, the aim of this study is to evaluate the biological protection (protective effect for liver, kidneys and spleen) effect of aqueous extract of *M. sativa* and their copper nanoparticales CuNPs against inflammation induced by phenylhydrazine.

- ✓ The phytochemical screening showed the richness of aqueous extract with phytocompounds like: polyphenols, flavonoïds, terpenoïds, saponins, rutin and naringin which may contribute to the plant being a source of treatment for many diseases.
- ✓ The in vitro study of aqueous extract and CuNPs appeared an important antioxidant and anti-inflammatory activities which may nominate these products to be an effective treatment for many diseases associated with oxidative stress and inflammation.
- ✓ The hematological analysis concluded that the rats treated by aqueous extract of *M. sativa* may have a positive impact in the hematopoietic system. Interestingly, there is a very clear effect treated by CuNPs, CuNPs showed protection against lipogenesis.
- ✓ The positive impact of by aqueous extract of *M. sativa*, and CuNPs on restoring some of biochemical markers that demonstrate the beneficial effect of treatment against the physiologic and metabolic alteration in several biological system related to our studying data.
- ✓ The action of aqueous extract and their CuNPs was observed in the decrease of MDA and increase of SOD levels which may give it the property of antioxidant activity.
- ✓ The high protection capacity of our treatment was extended to microscopic level which shows their high protection in cells stabilization against kidneys, spleen and hepato injury induced by phenylhydrazine. We shed the powerful effect of CuNPs and Alfalfa in our histological sections. This allows us to considered that these treatments have potential effect to limiting the histological alteration which associated with Anemia disease.

Perspective

For future studies, we hope that studies will focus more on detect all compounds of *M. sativa*, that can be more potent while testing them in inflammation, try to make extraction and the separation these compound , try to make a specific drug form CuNPs and *M. sativa*, Improve the MSE-CuNPs efficiency and evaluating the long-term effect of its use.

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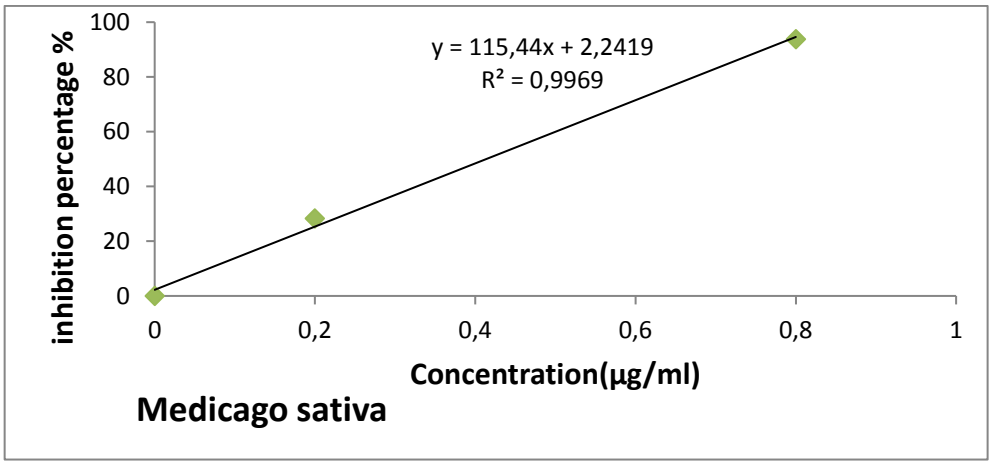
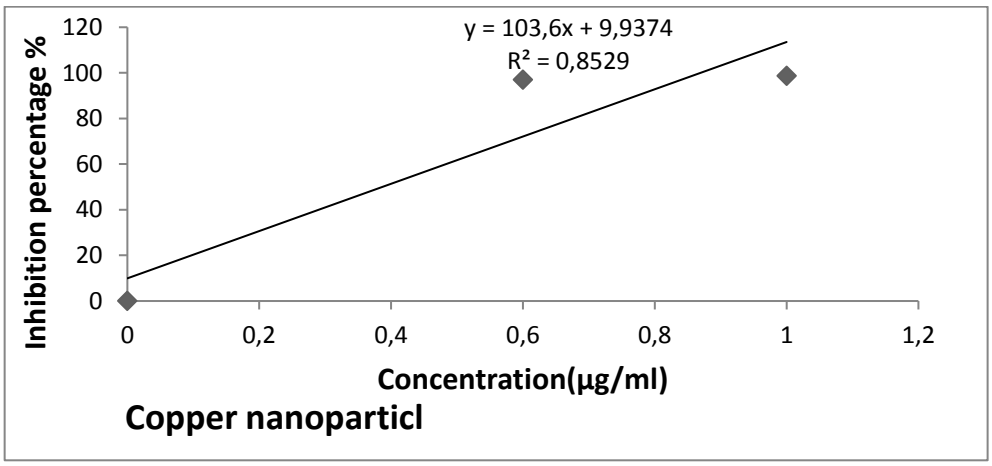
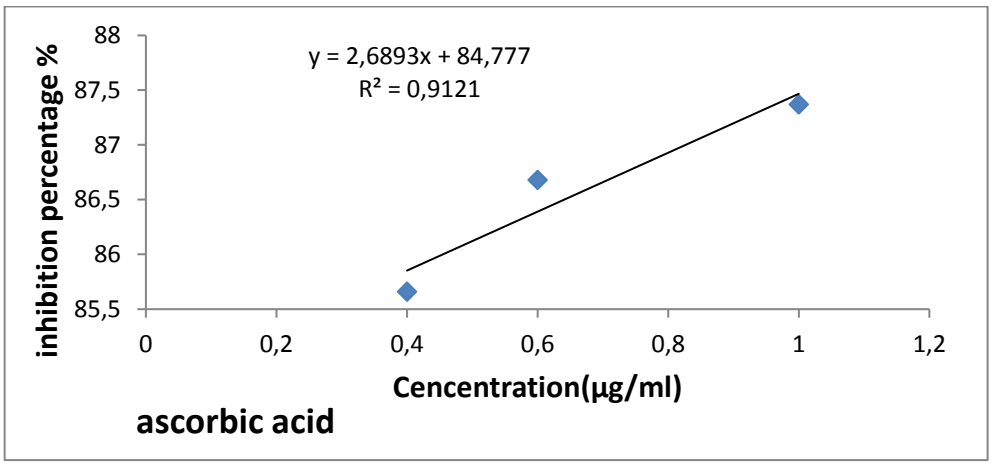
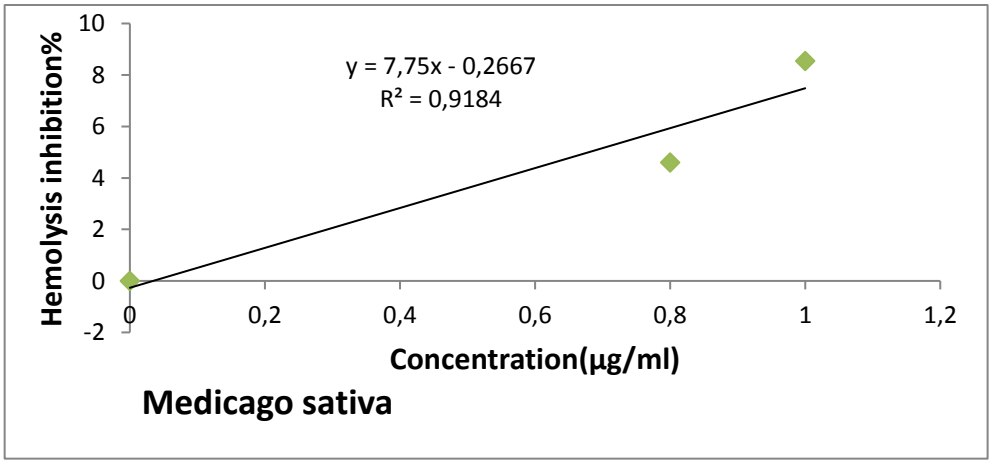
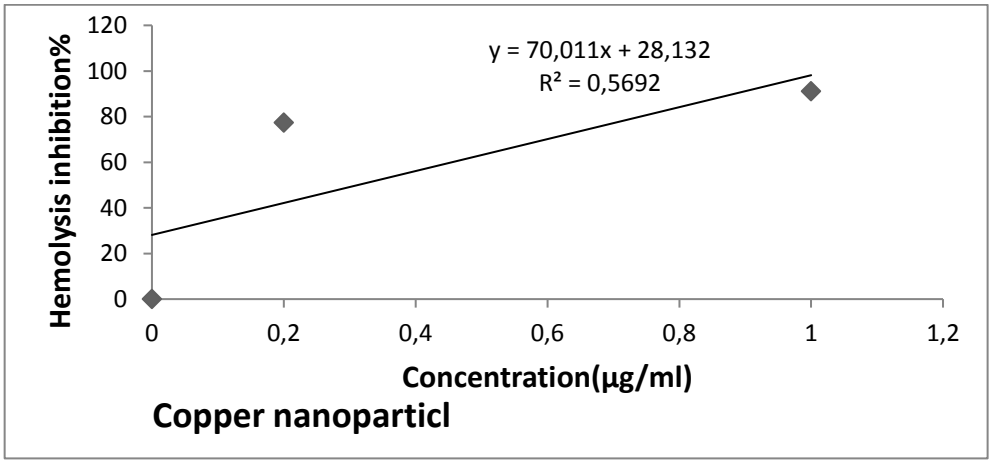
Annexes

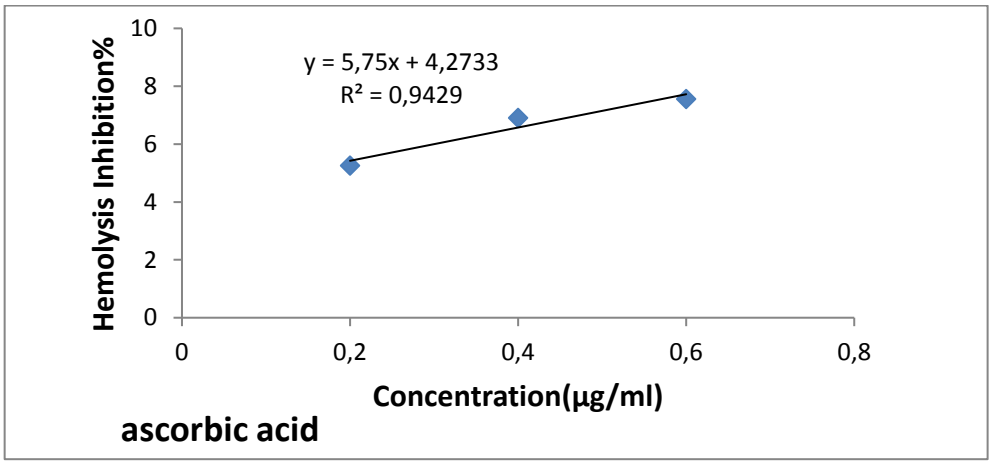

y = 115,44x + 2,2419
R² = 0,9969
inhibition percentage %
100
80
60
40
20
0
0
0,2
0,4
0,6
0,8
1
Concentration(µg/ml)
Medicago sativa


y = 103,6x + 9,9374
R² = 0,8529
Inhibition percentage %
120
100
80
60
40
20
0
0
0,2
0,4
0,6
0,8
1
1,2
Concentration(µg/ml)
Copper nanoparticl


y = 2,6893x + 84,777
R² = 0,9121
inhibition percentage %
88
87,5
87
86,5
86
85,5
0
0,2
0,4
0,6
0,8
1
1,2
Cencentration(µg/ml)
ascorbic acid


y = 7,75x - 0,2667
R² = 0,9184
Hemolysis inhibition%
Concentration(µg/ml)
Medicago sativa


y = 70,011x + 28,132
R² = 0,5692
Hemolysis inhibition%
Concentration(µg/ml)
Copper nanoparticl


y = 5,75x + 4,2733
R² = 0,9429
Hemolysis Inhibition%
Concentration(µg/ml)
ascorbic acid