الجمهورية الجزائرية الديمقراطية الشعبية
People's Democratic Republic Of Algeria
وزارة التعليم العالي و البحث العلمي
Ministry Of Higher Education And Scientific Research
University of Badji Mokhtar – Annaba جامعة باجي مختار-عنابة

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A thesis submitted for the fulfillment of Doctoral Degree (LMD) in Biological Sciences
Specialty: Animal Ecophysiology

Entitled

The protective effect of hawthorn in rats treated with a toxic dose of copper

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2020/2021

AKNOWLEDJMENT

Throughout the writing of this dissertation I have received a great deal of support and assistance.

First and foremost I am extremely grateful to my supervisors, **Prof. ABDENNOUR Cherif** for his invaluable advice, continuous support, and patience during my PhD study. His immense knowledge and plentiful experience have encouraged me in all the time of my academic research and daily life.

I would like to thank professor **KHELILI Kamel** to have accepted to examine this work I'm so grateful. Also many thanks to doctor **LOUDJANI Farida** and Doctor **CHETTOUM Aziez,** my deepest gratitude for the guidance you provided me as part of my dissertation committee.

TALBI Amina thanks for being the shoulder I always depend on.

DEDICATION

I dedicate this work to the memory of my dear father **REMITA Ahmed**; it was for you that I did

This journey and I regret that you are not with us at this long awaited moment. You are in my heart and my mind at all times.

Also I would like to thank my mother **REMITA Nora** for letting me through all the difficulties. I have experienced your guidance day by day. You are the one who let me finish my degree.

To my beloved husband **BOUDOUR Ali**, thank you for supporting me for everything, and especially I can’t thank you enough for encouraging me throughout this experience.

Thanks a bunch to my beautiful aunt **REMITA Fairouz Linda**.

ABSTRACT

The objective of this work is to assess the potential protective role of hawthorn *Crataegus monogyna*, a plant used in traditional medicine, against copper chronic intoxication. Male Wistar rats were divided into six groups; the control that received tap water and standard diet ad libitum, two positive controls treated respectively with Hawthorn leaves and fruits aqueous extract, a group treated with Cu and finally, two groups treated respectively with Cu+leaves and Cu+fruits. The treatment was done by gavage for 30 days. The reproductive, hepatic, renal, hematological, lipid and the oxidative stress markers were evaluated. Cu exposure reduced testosterone, sperm concentration, live sperm, VCL, VSL, VAP, ALH, BCF, GSH levels, and the activity of GPx activity compared to control groups. Dead sperm and MDA levels of testis, liver and kidneys were increased in rats of Cu group compared to the untreated control. When compared to the Cu group, levels of testosterone, sperm concentration, sperm motility, live sperm, VCL, VSL, VAP, ALH, BCF, GSH, and the activity of GPx were much higher significantly in the CuF and CuL groups along with a significantly lower MDA concentration. A significant increase in the activity of AST, ALT and ALP and the creatinine level of the Cu group were observed in Cu group compared to the control, but CuF and CuL have significantly decreased AST, ALT, ALP, creatinine and urea levels compared to the Cu group. Cu group has respectively increased the testis, hepatic and renal MDA concentration, and decreased GSH level and GPx activity compared to the control. The combined treatments (CuF and CuL) showed a significant decline in MDA concentration, accompanied with significant raise of GSH and GPx levels compared to the Cu group. In kidney, Cu group has respectively increased and decreased MDA concentration and the GSH and GPx activity, but CuF and CuL have significantly reduced the MDA concentration and raised both GSH level and GPx activity. Copper treatment reduced G6PD, RBC, HGB, HCT, MCV, TRIG and CHOL levels compared to the control. Compared to the Cu group, the two combined

treatments (CuL and CuF) have raised G6PD, RBC count, HGB, HCT, MCV, TRIG and CHOL levels, with a decrease in the number of WBC, PLT, and LDL levels. In conclusion, Cu administration to rats has induced reprotoxicity, hepatotoxicity and nephrotoxicity, but when hawthorn aqueous extracts of leaves and fruits were co-administrated with this metal, normal levels of most biological markers were established, while positive control (s) boosted sperm concentration and velocity (VCL and VAP).

Key words: Copper, sperm, hawthorn, reprotoxicity, hepatotoxicity, nephrotoxicity.

RESUME

L'objectif de ce travail est d'utiliser l'aubépine *Crataegus monogyna*, une plante utilisée en médecine traditionnelle, comme agent protecteur contre l'intoxication chronique au cuivre. Les rats mâles Wistar ont été divisés en six groupes; le contrôle ayant reçu de l'eau du robinet et un régime standard *ad libitum*, deux contrôles positifs traités respectivement avec des extraits aqueux de feuilles et de fruits, un groupe traité au Cu et enfin, deux groupes traités avec des feuilles + Cu et des fruits + Cu. Le traitement a été effectué par gavage pendant 30 jours. Les marqueurs reproductif, hépatique, rénal, hématologique, lipidique et du stress oxydatif ont été évalués. L'exposition au Cu a réduit les niveaux de testostérone, de spermatozoïdes, de spermatozoïdes vivants, de VCL, de VSL, de VAP, d'ALH, de BCF, de GSH et de GPx par rapport aux groupes témoins. Les taux de spermatozoïdes morts et de MDA testiculaire, hépatique et rénal ont augmenté chez les rats du groupe Cu par rapport au témoin. Par rapport au groupe Cu, les niveaux de testostérone, la concentration de spermatozoïdes, la motilité des spermatozoïdes, les spermatozoïdes vivants, VCL, VSL, VAP, ALH, BCF, GSH et l'activité de la GPx étaient beaucoup plus élevés dans les groupes CuF et CuL, avec une MDA significativement plus faible.

Une augmentation significative de l'activité de l'AST, de l'ALT et de l'ALP et du taux de créatinine du groupe Cu a été observée par rapport au témoin, mais CuF et CuL ont significativement diminué les niveaux d'AST, d'ALT, d'ALP, de créatinine et d'urée par rapport au groupe Cu. Le groupe Cu a respectivement augmenté les concentrations de MDA testiculaire, hépatique et rénale, et diminué le taux de GSH et l'activité GPx par rapport au témoin. Les traitements combinés (CuF et CuL) ont montré une baisse significative de la concentration de MDA, accompagnée d'une augmentation significative des niveaux de GSH et de GPx par rapport au groupe Cu. Dans le rein, le groupe Cu a respectivement augmenté et

diminué la concentration de MDA et l'activité GSH et GPx, mais CuF et CuL ont considérablement réduit la concentration de MDA et augmenté à la fois le niveau de GSH et l'activité GPx. Le traitement au cuivre a réduit les niveaux de G6PD, nombre de GR, HGB, HT, VGM, TRIG et CHOL par rapport au témoin. Par rapport au groupe Cu, les deux traitements combinés (CuL et CuF) ont une augmentation des niveaux de G6PD, GR (globule rouge), HGB (hémoglobine), HT (hématocrite), VGM (volume globulaire moyen), TRIG et CHOL, avec une diminution des taux de GB (globule blanc), PLT (plaquette) et LDL.

En conclusion, l'administration de Cu à des rats a induit une reprotoxicité, une hépatotoxicité et une néphrotoxicité, mais lorsque des extraits aqueux d'aubépine fruits et feuilles ont été co-administrés avec ce métal, des taux normaux de la plupart des marqueurs biologiques ont été établie, tandis que les témoins positifs ont augmenté la concentration et la vitesse des spermatozoïdes (VCL et VAP).

Mots clés: Cuivre, sperme, aubépine, reprotoxicité, hépatotoxicité, néphrotoxicité.

الملخص

تهدف هذه الدراسة الى استعمال الزعرور البري *Crataegus monogyna* ، نبات يستخدم في الطب التقليدي، كعامل وقائي ضد التسمم المزمن بالنحاس. قسمت الجرذان ويستار إلى ست مجموعات. المجموعة الشاهدة و التي تلقت ماء الصنبور والنظام الغذائي القياسي والذي يتم الوصول إليه بحرية، كما عولجت المجموعتان الإيجابيتان على التوالي بالمستخلص المائي لكل من الأوراق والفاكهة، بالإضافة إلى مجموعة النحاس (Cu) ومجموعتان عولجتا بالمستخلص المائي للأوراق مع النحاس CuL والمستخلص المائي للفاكهة مع النحاس CuF. أعطيت هذه المواد للجرذان بالتزقيم لمدة 30 يوما أين قيمت مؤشرات الإجهاد االتاكسدي، التكاثرية ، الكبدية ، الكلوية ، الدموية والدهنية.

أدى التعرض للنحاس إلى خفض مستويات هرمون التستوستيرون وتركيز الحيوانات المنوية والحيوانات المنوية الحية و VCL و VSL و VAP و ALH و BCF و GSH و GPx مقارنة بالمجموعة الشاهدة. كما زادت الحيوانات المنوية الميتة ومستويات MDA في جرذان مجموعة النحاس مقارنة بالمجموعة الشاهدة. عند مقارنتها بمجموعة النحاس ، كانت مستويات هرمون التستوستيرون، وتركيز الحيوانات المنوية والحيوانات المنوية الحية، وحركة الحيوانات المنوية ، و VCL ، و VSL ، و VAP ، و ALH ، و BCF ، و GSH ، و GPx أعلى بكثير في مجموعات CuF و CuL ، إلى جانب انخفاض تركيز MDA بشكل ملحوظ.

لوحظت زيادة ملحوظة في نشاط AST و ALT و ALP ومستوى الكرياتينين في مجموعة Cu مقارنةً بالمجموعة الشاهدة، لكن Cu+F و Cu+L أدت إلى انخفاض كبير في مستويات AST و ALT و ALP والكرياتينين واليوريا مقارنةً بمجموعة النحاس. زادت مجموعة النحاس من تركيز MDA الكبدي والكلوي، وخفضت مستوى GSH ونشاط GPx على التوالي مقارنةً بالمجموعة الشاهدة. أظهرت المجموعتان (Cu+F و Cu+L) انخفاضًا كبيرًا في تركيز MDA ، مصحوبًا بارتفاع كبير في مستويات GSH و GPx مقارنة بمجموعة Cu ، وكذلك أظهرت كل من المجموعتان الإيجابيتان (F و L) زيادات كبيرة من مستويات

GSH و GPx مقارنة بالمجموعة الشاهدة. في الكلى، زادت مجموعة Cu تركيز MDA وأنقصت في نشاط GPx على التوالي، لكن Cu+F و Cu+L قللت بشكل كبير من تركيز MDA ورفعت مستوى GSH ونشاط GPx. خفض النحاس مستويات G6PD و RBC و HGB و HCT و MCV و TRIG و CHOL مقارنةً بالمجموعة الشاهدة. وبالمقارنة مع مجموعة Cu، فإن المجموعتان (CuL و CuF) أدت إلى زيادة في مستويات G6PD و RBC و HGB و HCT و MCV و TRIG و CHOL، مع انخفاض في مستويات WBC و PLT و LDL.

في الختام ، أدى إعطاء النحاس للفئران إلى حدوث تسمم تناسلي ، كبدي ، وكلوي ، ولكن عند إضافة مستخلصات الزعرور المائية مع هذا المعدن، أدى إلى الإقلال من سمية النحاس بالمحافظة على مستويات طبيعية لمعظم المؤشرات البيولوجية ، أما في الشواهد الايجابية فزاد تركيز الحيوانات المنوية وسرعتها (VCL و VAP).

الكلمات المفتاحية: النحاس ، السائل المنوي، الزعرور ، السمية التناسلية ، السمية الكبدية ، السمية الكلوية.

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ABBREVIATION LIST

Name	Abbreviation
Cu	Copper
CuSO4	Copper sulfate
VCL	Curvilinear velocity
VSL	Straight line velocity
VAP	average velocity
ALH	Amplitude of lateral head displacement
BCF	Beat cross frequency
GSH	Glutathione
GPx	Glutathione Peroxidase
MDA	Malondialdehyde
AST	Aspartate Transaminase
ALT	Alanine aminotransferase
ALP	Phosphatase alcaline
G6PD	Glucose-6-phosphate dehydrogenase
RBC	Red blood cell
HGB	Hemoglobin
HCT	Hematocrit
MCV	Mean corpuscular volume
PLT	Platelets
CHOL	Cholesterol
LDL	Low density lipoprotein
HDL	High density lipoprotein
CREA	Creatinine

Cu°	Copper(0): metallic copper
Cu^{+}	Copper(I): cuprous ion
Cu^{+2}	Copper(II): cupric ion
Ph	Potential hydrogen
DMT	Divalent metal transporter
CTR	Copper transporter
hCTR	Human copper transporter
ATP	Adenosine triphosphate
SOD	Superoxide dismutase
GnRH	Gonadotrophin-Releasing Hormone
LH	Luteinizing Hormone
FSH	Follicle-Stimulating Hormone
LHRH	luteinizing hormone-releasing hormone
H_2O_2	Hydrogen peroxide
OH°	Hydroxyl radical
AD	Alzheimer's disease
PD	Parkinson disease
CP	Ceruloplasmin
ROS	Reactive oxygen species
L	Leaves
F	Fruits
CASA	Computer Assisted Semen Analysis
CSA	Class sperm analyzer
ELISA	enzyme-linked immunoassay
TCA	Trichloroacetic acid
TBA	thiobarbituric acid
DTNB	5, 5'-dithio-bis-2-nitrobenzoic acid

GSSG	Glutathione disulfide
TBS	Tris and NaCl
EDTA	Ethylene Diamine Tetra Acetic Acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NaCl	Sodium chloride
Zn	Zinc
STAR	Steroidogenic acute regulatory protein
HMG-Co A reductase activity	3-hydroxy-3-methyl-glutaryl-CoA reductase

INTRODUCTION

Copper is an important trace element of numerous metalloenzymes that are involved in energy and antioxidant metabolisms. However, some of copper's chemical forms, such as copper sulfate are very toxic (Badiye et al., 2013). Copper binds to binding proteins in the bloodstream and, distributes to all tissues especially the brain and the liver; the latter can secrete excess of the metal into bile. Hypercupremia may cause several oxidation reactions, inflammations, and tissues damage through the generation of free radical (Sinkovic et al., 2008). Copper can enter the body orally through food, by inhalation or through the skin by direct contact. Copper is used in agriculture as a fungicide, herbicide, and insecticide (Blundell et al., 2003). Also, it is used as an electrical conductor in several industries; it has many chemical applications, and is known as a coinage metal. In nature, copper exposure may be caused by dusts, volcanoes and forest fires. Copper dyshomeostasis has been linked to a variety of disorders, for example, ATP7A and ATP7B are both involved in copper metabolism; in which the mutations of the former may lead to Menkes' disease, but that of the latter causes Wilson's disease (Mario et al., 2014).

Long term exposure of rats to copper leads to its accumulation in liver, followed by kidney and brain, in which free tissue copper was positively correlated with oxidative stress and organs' dysfunction (Kumar et al., 2016). Liver is known to play a key role in maintaining Cu homeostasis, and also the xenobiotics' detoxification (Chiang, 2014), since all absorbed nutrients pass into this vital organ by the portal vein.

High copper concentration may provoke many pathological conditions (Parmar et al., 2002) as anemia by the destruction of red blood cells (DES, 2013), abnormal lipid profile (Burkhead and Lutsenko, 2013) and lower triglycerides concentrations (Wuolikainen et al., 2014). Furthermore, high copper load provoke cell injury by oxidizing cell membranes

(Saravu et al., 2007), mitochondrial dysfunction and lowering antioxidant enzymes, leading to oxidative stress damage (Tiwari et al., 2018).

According to Wong et al. (2001), a positive correlation between blood Cu concentration and sperm motility dysfunction was found. However, cytosolic Cu is mainly bound to metallothioneins that may reduce its toxicity to some extent. The hydroxyl free radicals induced by the Fenton reaction of copper are very destructive to tissues (Moriwaki et al., 2008) of the testis and epididymis, and also reduce antioxidant biomarkers such as catalase, superoxide dismutase, glutathione, and glutathione peroxidase. This oxidation can change sperm quality by modifying spermatozoa shape and movement. In addition, copper's effect on the pituitary receptor can provoke hormonal imbalance.

Nowadays, interest of using plant compounds has been growing faster in worldwide due to their benefits on health (Nandi and Ghosh, 2016). Hawthorn, *Crataegus monogyna*, is one of very common shrub plant used in traditional medicine and even in modern medicinal treatments (Fong & Bauman, 2002), which considered a relatively safe herb and without serious adverse effects (Zapfe, 2001). *C. monogyna* is well distributed in the Mediterranean region and is rich in proanthocyanidins and flavonoids (Bahorun et al., 1996), which are superoxide anion (Keser et al., 2014), hydroxyl radical, hydrogen peroxides scavengers and lipid peroxidase reducer (Rice-Evans, 2004) that make it a powerful antioxidant (Yao et al., 2008). Interestingly, flavonoids of hawthorn have the ability to inhibit copper intake (Kuo et al., 1998).

The objectives of this work are to study the ability of the common *C. monogyna* aqueous extract of both fruits and leaves in protecting male wistar rat from copper-induced toxicity by investigating reproductive, hepatic, renal, hematological, lipid, and oxidative stress markers.

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CHAPTER 1

LITERATURE REVIEW

LITERATURE REVIEW

COPPER

Heavy metals are increasing in our life by industrial, agricultural and domestic discharges that can cause several environmental health problems. Copper was one of the first metals used by human, it occurs in nature in its metallic form, it was first discovered in Anatolia (Turkey) and Spain back nearly to 5000 BC (Stern et al., 2007).

Copper is a member of the third transition series of elements, with atomic number and atomic weight being 29 and 63.546, respectively. There are two stable copper isotopes, ^{63}Cu and ^{65}Cu, and these have natural abundances of 69.2 and 30.8%, respectively. Radioactive isotopes include ^{64}Cu (t½ = 12.7 h) and ^{67}Cu (t½ = 61.9 h) (Stern et al., 2007). According to Saravu et al., (2007), copper sulfate is known as Blue Vitriol or Blue Stone, and forms bright blue crystals containing 5 molecules of water [CuSO4. 5H2O]. Copper sulfate is the most common copper salt; however, other important copper salts include carbonate, cyanide, oxide and sulfide (Rosmarie, 1992). It has a nauseous and metallic taste. Copper solutions are acid to litmus, and freely soluble in water (Barceloux, 1999). Copper has the following characteristics:

• Copper(0): metallic copper; Cu°.

• Copper(I): cuprous ion; Cu+, unstable at neutral pH, oxidized to Cu2+ in the air.

• Copper(II): cupric ion; Cu2+, stable, forms Cu(OH2) in water at alkaline pH.

- Copper also exists in the trivalent form [copper(III)], but this form is rare.

• Copper is an essential component of metalloenzymes, where copper participates in redox reactions by cycling between copper(I) and copper(II) oxidation states.

• It is essential structural component of macromolecules, by providing the appropriate coordination chemistry to maintain higher order structure (Stern et al., 2007).

The World Health Organization (WHO) categorizes a metal as essential when "absence or deficiency of the element from the diet produces either functional or structural abnormalities and that the abnormalities are related to, or a consequence of, specific biochemical changes that can be reversed by the presence of the essential metal (WHO, 1996). Copper is transported by ceruloplasmine that can carry 90% of blood copper (Walshe, 2007). Copper play a role in iron homeostasis as a cofactor in ceruloplasmin (Ralph & McArdle, 2001).

Copper is widely involved in several biological processes such as cell respiration, maturation of erythrocytes (Abbaoui et al., 2017), and act as a cofactor of several enzymes including superoxide dismutase (Cu, Zn-SOD), dopamine monooxygenase, and cytochrome oxidase (Turnlund, 1999).

On the contrary, copper can be toxic to living organisms when it reaches a high concentration (liu et al., 2016).

1- Sources of copper

Copper as a food is found in vegetables such as potato, and legumes such as beans and peas. Nuts, including peanuts, pecans, mushrooms are rich and some grains (IOM, 2001), wheat, rice, lemons and raisins (Stern et al., 2007). Based on the results of the US Department of Agriculture 1989– 1991 survey of food consumption, about 40% of dietary copper comes from yeast breads, white potatoes, tomatoes, cereals, beef and dried beans and lentils (Subar et al., 1998). Mollusks are rich in copper (Abbas et al., 2018), which can have up to 5 mg/kg wet weight (Stern et al., 2007). Shellfish with hemocyanin as a respiratory pigment also is an important source of copper, in addition to animals' liver (Stern et al., 2007).

However, mining, smelting and refining of copper, industries producing copper products such as wire, pipes and sheet metal, and fossil fuel combustion are the important environmental sources (DES, 2013). Water pipes are often made of copper and bath fixtures may be made from brass and bronze alloys that contain copper lead to higher copper concentration in

drinking water (DES, 2013). Other releases of copper to the environment include agricultural use against plant diseases and treatments applied to water bodies to eliminate algae (DES, 2013).

2- Distribution in the environment

In the atmosphere, 0.4% of copper comes from wind dispersion of particulate matter from smokestack emissions (Barceloux, 1999). Surface water, groundwater, seawater and drinking water contain different copper concentrations as complexes or particulate matter forms (ATSDR, 2002). Copper concentration in drinking water depends on pH, hardness and copper availability in the distribution system (WHO, 2004).

3- Absorption

It was reported that copper intake form food is 1.54-1.70 mg/day for men and 1.13-1.18 mg/day for women, where 13% of the USA population consumes copper supplements (Food and Nutrition Board, 2001). As copper is soluble in water, it makes water as an important source of this metal (NAS, 2000). The total body content of copper is 150 mg (Metals and Related Compounds, 1997).

In mammals; 24% to 60% of copper is absorbed in the stomach and the small intestine (DES, 2013), where the percentage of absorption differs among species (Stern et al., 2007). In rats, copper is absorbed in the duodenum (van Campen and Mitchell, 1965) and from the lower small intestine in hamsters (Crampton et al., 1965). The site of maximal copper absorption is not known for humans, but it is assumed to be the stomach and upper intestine because of the rapid appearance of ^{64}Cu in the plasma after oral administration (Bearn and Kunkel, 1955).

Inhalation of copper generally happened by accident or suicide, also dermal exposure is very low (DES, 2013).

Zinc, iron, molybdenum, lead and cadmium are known to be copper antagonists (Cousins, 1985), while zinc is a powerful inhibitor of copper absorption in the intestine (Mandil et al., 2016), possibly by competing with copper for transport and/or by increasing intestinal metallothionein concentrations. Metallothioneins are a group of small, heavy-metal binding proteins that serve in detoxification and metal buffering (Suzuki et al., 2002). Fructose and other carbohydrates, dietary cellulose fiber, and phytate were found to reduce the bioavailability of copper (Wapnir, 1998). Citrate, phosphate, and glutamate can form copper complexes that increase absorption.

4- Distribution in the body

Beside to amino acids, albumin is a protein that can bind to absorbed copper in the portal blood and enter to the liver by specific membrane transporters, while it is incorporated into ceruloplasmin, which contain greater than 95% of copper and later released into the plasma (Rosmarie, 1992). In the mucosal cells, copper binds generally to metallothionein or glutathione (Tapiero et al. 2003). In serum, about 98% of copper bound to ceruloplasmin in association with albumin (Roychoudhury et al., 2016). When copper reach to high concentration, it binds mainly to albumin rather than to ceruloplasmin (Piscator, 1979). Copper distribution to other tissues depends on the amount of dietary intake (Rosmarie, 1992).

In mammals, copper is absorbed across the apical brush border membrane of the mucosal cells, while researchers found the existence of an ATP-driven high-affinity copper uptake system in the brush border membrane (Knpfel et al., 2005). Copper transported by the passive diffusion (Goode et al., 1989) and released from intestinal cells to move to the serosal capillaries, where it binds to albumin, glutathione, and amino acids in the portal blood (Bligh et al., 1992).

Once copper is in the intestine, it can pass through the divalent metal transporter 1 (DMT1) or also called (DCT1) (Stern et al., 2007), in which the treatment with DMT1 antisense oligonucleotide may inhibit 48% of copper uptake (Arredondo et al., 2003), in addition, copper-transport protein CTR located at the brush border membrane showed a high affinity to copper (Zhou and Gitschier, 1997).

The hCTR is likely to be the major copper uptake system of all cells, except perhaps for enterocytes (Lee et al., 2002a). The hCTR was expressed in all organs and tissues examined, with liver, heart, and pancreas exhibiting the highest levels, but brain and muscles the lowest, and the intestine expressing intermediate levels. A second human copper transport protein, hCTR2, was also identified (Zhou and Gitschier, 1997), but its function remains unclear at this time. CTR1 does not appear to require ATP for copper transport, but the driving force for copper uptake into cells remains unclear (Lee et al., 2002a). More recent findings suggest that hCTR1-mediated copper uptake into mammalian cells is regulated by a posttranslational mechanism involving copper-stimulated endocytosis and degradation of the transporter (Petris et al., 2003).

Copper is stored mainly in liver, kidney, brain and muscles (Roychoudhury et al., 2016). The total amount of copper in an adult is estimated to be about 90–110 mg. The organs with the highest concentrations are the liver, brain, kidney and heart (Thiele et al., 2003). Bones and skeletal muscles contain about 47% and 27% of the copper, respectively (Linder, 1991). Brain concentrations range from 3.1 to 5.1 mg/g wet weight (Davies et al., 2014). Within the brain, the distribution of copper is heterogeneous. Concentrations are higher in the hippocampus, substantia nigra and locus coeruleus (Hung, et al., 2013). Glial cells are enriched in copper as compared to neurons (Scheiber et al., 2014).

5- Metabolism

Copper is mainly absorbed in the duodenum and proximal jejunum, with a little bit of absorption occurring in the stomach and the distal portion of the small intestine (Hordyjewska et al., 2014). The human copper transport protein 1 (hCTR1), located at the level of enterocytes, transfers the ion following the reduction of dietary Cu2+ into Cu+. In hepatocytes, copper binds to metallothioneins (MTs), to reduced glutathione (GSH) or to one of the copper chaperones regulating the traffic of intracellular copper (CCS: chaperone for superoxide dismutase 1 SOD1, which is the sole cytosolic cupro-enzyme; COX17: chaperone for cytochrome C oxygenase; ATOX1 antioxidant-1: chaperone for the ATPases, ATP7A and ATP7B). The group of trans-membrane copper transporters includes CTR1, ATP7A and ATP7B. ATP7A (expressed in the placenta, gut and nervous system) and ATP7B (expressed in the hepatocytes, where it exports copper into the bile and provides copper to nascent ceruloplasmin, and in the nervous system) are linked to the enzyme, tyrosinase, and the ceruloplasmin, respectively (Manto, 2014).

6- Excretion

Under normal physiological conditions, approximately 98 % of the Cu excretion is made through the bile and the remaining 2 % is through the urine (Wijmenga and Klomp 2004). The biliary Cu is excreted mainly in the feces (Poujois and Woimant, 2018), but resorption of biliary Cu is negligible (Farrer andMistilis, 1967). Biliary Cu excretion following administration does not increase proportionally with dosage, suggesting that the hepatobiliary transport of copper is saturable (Gregus andKlaassen, 1986). Small amounts of Cu are secreted daily by salivary, gastric, pancreatic and duodenal excretions (Roychoudhury et al., 2016). The dietary Cu biological half-life is reported to be 13 to 33 days, with biliary excretion being the main route of elimination (Barceloux, 1999). Copper deficiencies enhance

affinity of methallothionein in enterocytes for copper, thus increasing it absorption and vice versa (Nagral et al., 2018).

7- Toxicity

The usual routes by which humans receive toxic exposure to Cu are through skin or eye contact, as well as by inhalation of powders and dusts (USEPA, 1986). Copper sulphate being a corrosive acid, results in caustic burns of the esophagus, super cial and deep ulcers in the stomach and the small intestine (Chugh, 1977). Changes of acute gastritis, hemorrhages in the intestinal mucosa and necrosis have been reported (Papodayanakis et al., 1969). Copper sulphate has a metallic taste, and it is rated as moderately toxic for human, the occurrence of copper sulfate poisoning varies in different regions depending on availability of this toxic agent (Meena and Bansal, 2014). In case of poisoning, it is commonly consumed with suicidal intentions; however, accidental poisonings have been reported from children as well (Blundell et al., 2003).

7-1 Short-Term (Acute) Effects

Acute poisoning from ingestion of excessive copper can cause temporary gastrointestinal distress with symptoms such as nausea, vomiting, and abdominal pain. Liver toxicity was seen in doses high enough that resulted in death (DES, 2013). High levels of exposure to copper can cause destruction of red blood cells, possibly resulting in anemia (DES, 2013), methaemoglobinaemia, acute renal failure and oliguria (Agarwal et al., 1993). Symptoms generally appear after 15–60 min of exposure (Stenhammar, 1999). The acute lethal dose for adults lies between 4 and 400 mg of copper (II) ion per kg of body weight, based on data from accidental ingestion and suicide cases (Jantsch et al., 1984–1985).

7-2 Long Term (Chronic) Effects

Mammals have efficient mechanisms to regulate copper stores in the body; they are generally protected from excess dietary copper levels. However, at high levels, chronic over-

exposure to copper can damage liver and kidneys. Symptoms of liver toxicity (jaundice, swelling, pain) usually do not appear until adolescence (DES, 2013).

8- Clinical features

8-1 Hepatic

Copper can enter to body from daily diet and principally targeting the liver as the main organ for Cu metabolism, the liver cells use Cu for many metabolic processes such as respiration and antioxidant defense, also it has an important role in some proteins synthesis that contain copper: ceruloplasmin. Excess Cu in hepatic cells occur damages to these cells such as necrosis. Jaundice which was noticed after 24-48h of high Cu intake (Singh and Singh,1968) may provoke oxidative stress to erythrocytes and cause RBC hemolysis (Barceloux et al., 1999) that has been described in patients with Wilson's disease (Nederbragt et al., l984). High Cu intake might affect hepatocytes and it is associated with hepatomegaly (Saravu et al., 2007).

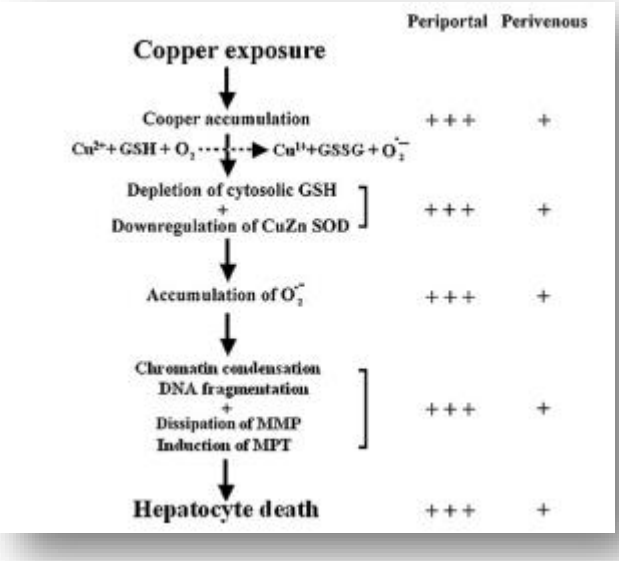

Figure 1: schematic representation of biochemical basis for the regional differences in copper-mediated hepatotoxicity (Roy et al., 2009).

In the liver, Cu mostly binds to metallotionein. It is assumed that Cu that is not bound to metallothionein or stored in any form in lysosomes and cannot be transported out of the cell will remain in. It was suggested that liver cell damage may be caused by Cu-mediated lipid peroxidation (Indquist, 1968).The ability of Cu to catalyze the peroxidation of unsaturated fatty acids and the generation of hydroxyl radicals, and lysosomal membrane damage may be caused by lipid peroxidation or binding of Cu to the membrane sulphydryl groups (Nederbragt et al., l984).

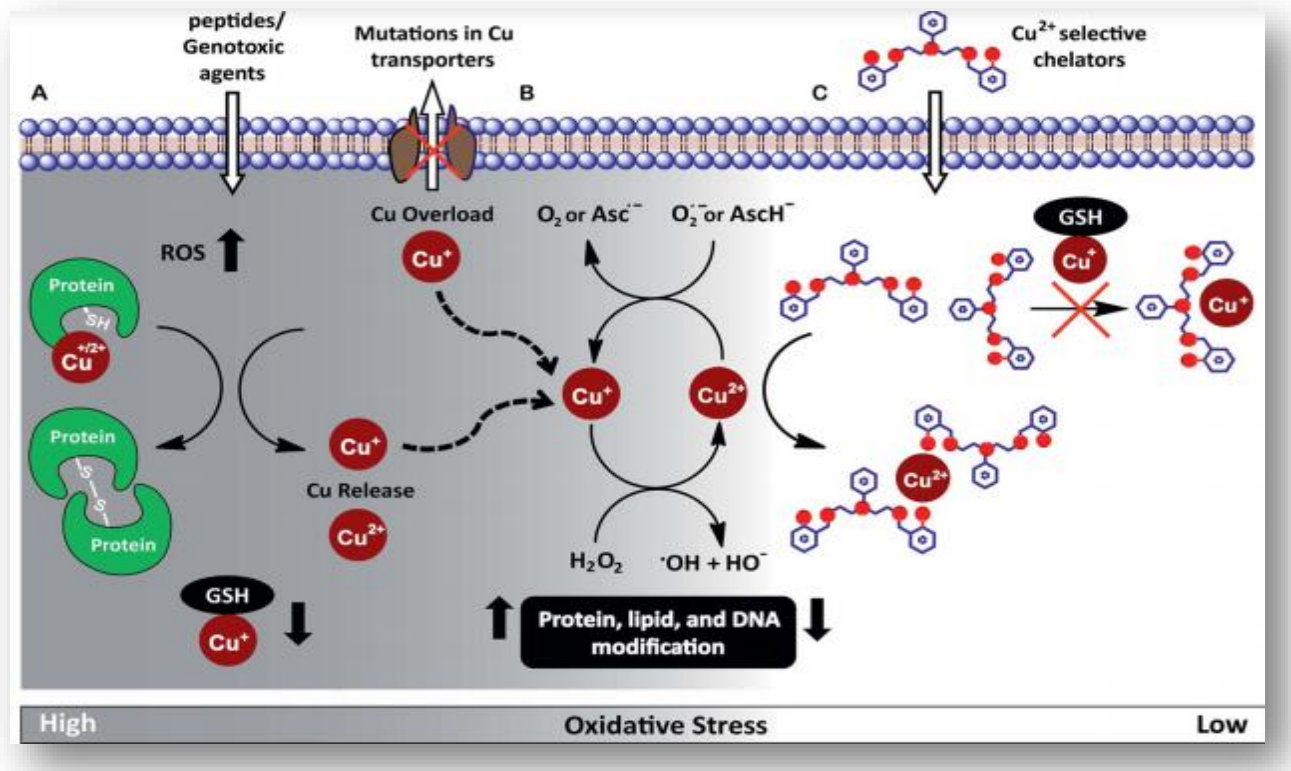

Figure 2: (A) scheme highlighting mechanisms that can increase intracellular Cu ion levels. Exposure to external agents and misfolded peptides can cause increased levels of ROS species, leading to oxidative stress. Increased oxidative stress leads to protein oxidation releasing Cu ions from proteins and also reduces levels of glutathione (GSH) bound Cu+. Mutations in Cu ion transporters lead to elevated intracellular Cu ion levels. (B) Cu+/Cu2+ catalyzed Fenton reaction produces reactive hydroxyl radicals, HOc. Cu2+ is reduced by cellular reductants like superoxide (O2c) and hydroascorbate (AscH) to complete the catalytic cycle. (C) Proposed cell-permeable Cu2+ chelators that alleviate oxidative stress via selective Cu2+ chelation (Rakshit et al., 2018).

8-2 Nephrotic

After 48h of copper sulfate poisoning, renal complications were observed (Mehta et al., 1985) and acute renal failure was noticed in 20-40% of patients (Dash, 1989). Copper may damage renal cells and provoke tubular necrosis, and tubular contained hemoglobin (Saravu et al., 2007). Some markers maybe appear in urine after copper intoxication such as oliguria, anuria, albuminuria, hemoglobinuria, and hematuria (Mehta et al., 1985) are indicators of renal dysfunction.

8-3 Nervous system

Copper enriched in synapse have a signaling role (Gaier et al., 2013), while it is found in SOD3 of secretary granules, constitutive vesicles and endosomes (Manto, 2014). The interaction of copper with both glutamatergic and GABAergic synapses activates voltage-gated calcium channels (Gaier et al., 2013).

Copper deficiency can induce neurodegenerative alterations such as Parkinson's diseases (Dusek, et al., 2015); it may acts as a cytotoxic agent altering DAergic neurons within the globus pallidus and the basal ganglia, which is known by primary motor dysfunction, rigidity, bradykinesia, walking difficulty, ataxia and hypokinesia (Abbaoui et al., 2017).

In case of high c Cu levels, data showed that copper causes a loss of TH-immunoreactivity on the midbrain nuclei of the DAergic including SNc and VTA disruption, as well as a decrease of TH- immunopositive fibers density in the dorsal striatum (Abbaoui et al., 2017). Also Cu may induce damages to the innervations of the DAergic system (Abbaoui et al., 2016), and causes neocortical neurons apoptosis (Sheline et al., 2002) and neural cells death (Südmeyer et al., 2006).

8-4 Cardiac

The vascular system integrity and structure is related to copper, when copper leads to the production of lysyl oxidase that is important in the formation of elastin and collagen cross-

linking (David and Watts, 1989). Copper deficiency may induce vascular weakness as aneurysms, heart enlargement, heart failure, and infarcts (David and Watts, 1989), also it may be related to ischemic heart diseases (kelvay1975). Copper toxicity may generate cardiovascular collapse, hypotension and tachycardia (Wahal et al., 1963). Methemoglobinemia provoked by copper can result cardiac dysrhythmia and hypoxia (Price, 2002), in addition it may leads to vascular and cardiac cells sepsis caused by transmucosal invasion (Nelson, 2002).

8-5 Reproduction

Component of the environment may alter the physiological regulations including reproductive health by decreasing semen quality, subfertility etc (Swan et al., 2000). Moreover, certain compounds have direct effect on the pituitary function by altering the hormones releasing, and indirect effect by modifying the stimulation of the central nervous system and gonadal hormones effects (Cooper et al., 1986). Copper may give rise to a decline in male reproductive capacity (Sakhaee et al., 2012).

Copper plays an important role in the activity of dopamine β-monooxygenase by catalyzing hydroxylation of dopamine to noradrenaline, which is an essential neurotransmitter involved in the secretion of gonadotropin releasing hormone (GnRH). Binding of GnRH with a specific receptor on the gonadotrope cell membrane is responsible for the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary (Michaluk and Kochman, 2007). Complexes of Cu with GnRH reportedly evoked the release of FSH more effectively than LH (Kochman et al., 2005), but complex of Cu2+ with luteinizing hormone releasing hormone (LHRH) brought about a high release of LH and even higher release of FSH (Yu et al. 2008).

Cu induces modifications on quality of spermatozoa and testicular histopathology (Sakhaee et al., 2012). The primary functions of the testicles are to produce spermatozoa,

androgens, and male sex hormone (testosterone) (Forgacs et al., 2012). Copper was found to play an essential role in spermatogenesis and male infertility in Wistar rats (Sakhaee et al., 2012). Copper intake even with low dose showed adverse effects on testis morphology (Babaei et al. 2012) by lowering the sperm count and spermatozoa head abnormalities, while higher dose compromised spermatozoa tail membrane integrity, viability and motility (Schramm et al., 2014). Among men, symptoms of adverse effect of Cu usually include prostate enlargement, prostate infections, erectile dysfunction, depression, anxiety, testicular pain and testicular cancer (Badiye et al., 2013). At any stage of cell differentiation the disruption of spermatogenesis may result in the decrease of total sperm count (Sharpe et al., 2003). Seminal plasma Cu concentrations found in oligozoospermic, asthenozoospermic and azoospermic groups was significantly higher than normozoospermic group (Eidi et al., 2010).

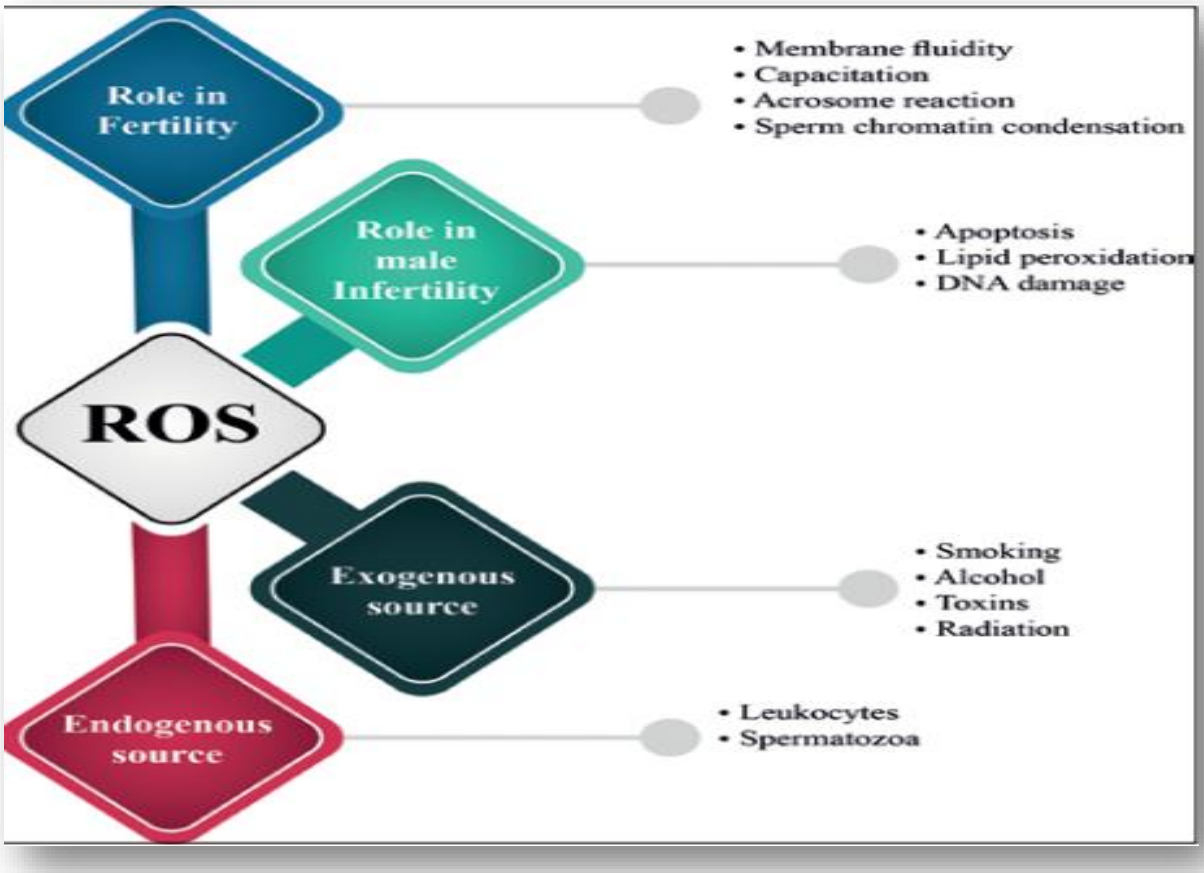

Figure 3: roles of ROS in fertility and infertility (Bardaweel et al., 2018).

8-6 Hematology

The presence of copper in red blood cells is known as erythrocuprein (Cartwright et al., 1957). Some pathology is found in the blood cells in the presence of high toxic dose of copper as the following:

- Copper in blood taken by erythrocytes can induce oxidative stress and hemolysis of RBCs (Barceloux, 1999).
- Intravascularhemolysis occurs after 12- 24h of ingestion (Saravu et al., 2007).
- Methemoglobinemia occurs early in the patients' clinical course and is rapidly followed by hemolysis (Saravu et al., 2007).
- Coagulopathy can occur due to liver injury or direct effect of free copper ions on the coagulation cascade (Nelson, 2002).
- Copper inhibits the sulfhydryl groups on enzymes in important antioxidant systems including G-6-PD and glutathione reductase, reducing their free radical scavenging activities (Aggrawal, 2016). Intravascular hemolysis is caused by the inhibition of G-6-PD (Aggrawal, 2016).

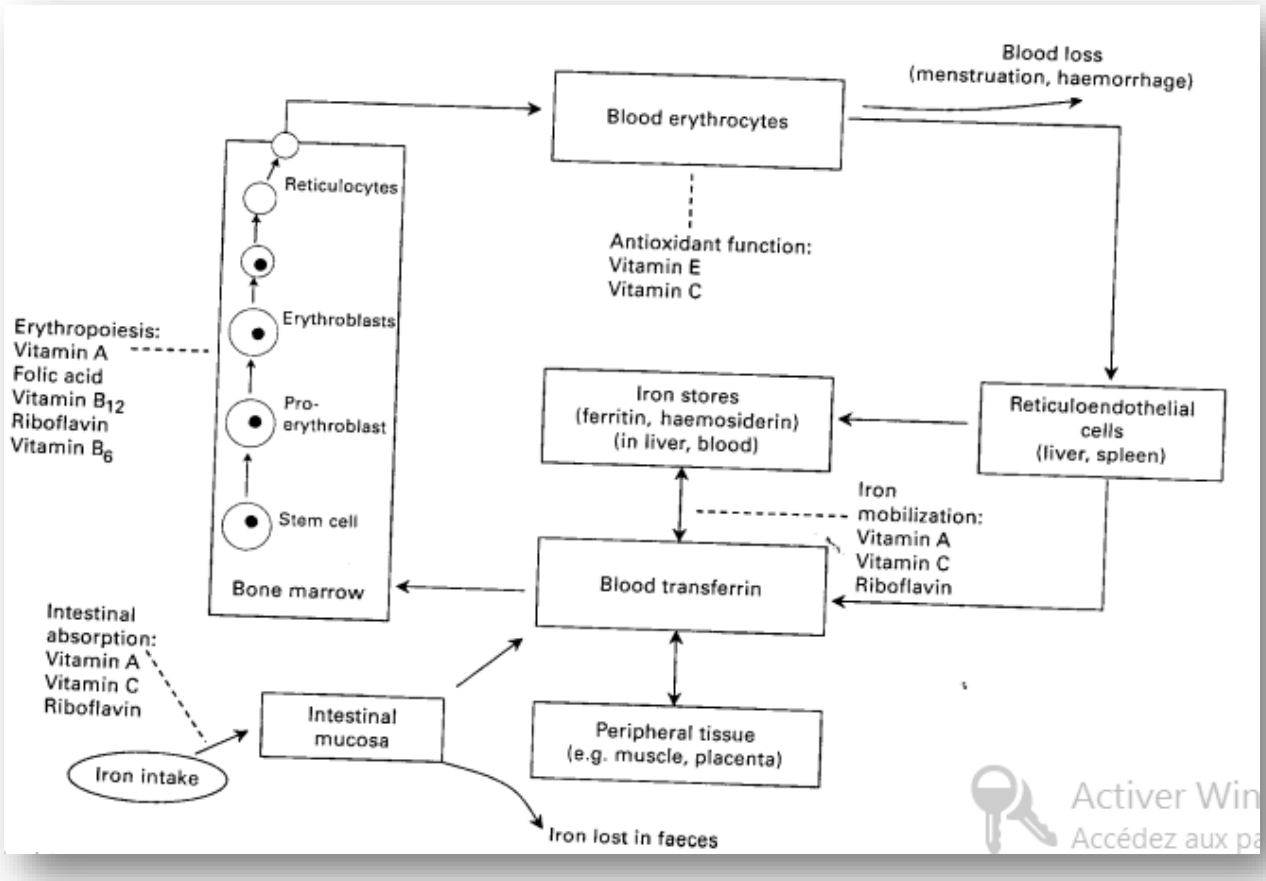

Figure 4: roles of vitamins in iron metabolism and erythropoiesis (Hughes-Jones and wickramasinghe, 1996).

8-7 Lipid profile

Copper is a redox-active metal leads to cells toxicity caused by the imbalance in metal homeostasis by generating free radicals (Sayre et al., 1999). Dysfunction of cells leads to lipid peroxidation and inactivates receptors and changes ion permeability (Ozcelik et al., 2003), and the increase of Cu intracellular levels activates hemolytic cleavage of H_2O_2 yielding HO° and phospholipid peroxidation (Rosario et al., 2017).

Deficiency of copper showed a decrease in high density lipoprotein (HDL) and an increase in low density lipoproteins (LDL) (Klevay et., 1971). While copper toxicity gives rise to hypercholesterolemia (David and Watts, 1989) and lowering the triglycerides (Curti et al., 2011).

9- Copper-related diseases

9-1 Wilson disease (WD)

Wilson disease is characterized by an abnormal accumulation of copper in the liver and brain (Poujois and Woimant, 2018). It is an autosomal recessive disorder of copper metabolism resulting from insufficient copper excretion into biles (Thomas et al., 1995). Binding copper to ATP7B causes ATP hydrolysis, which is the main energy source for copper transportation. This gene codes for a copper transporting ATPase, located preferentially in the liver but also in the brain (Poujois and Woimant, 2018). The mutation of ATP7B then could lead to a decrease in the synthesis of copper-bound ceruloplasmin (Nagral et al., 2018).

9-2 Alzheimer's disease (AD)

Free plasma levels of copper increase with ageing (Noda et al., 2013). Metal dyshomeostasis might impact not only on AD onset, but also on its progression (Squittiand Polimanti, 2013). The ratios of plasma/serum copper levels are significantly higher in AD (Ventriglia et al., 2012). Copper not bound to ceruloplasmin, rather than absolute serum copper levels, is a key-concept for the understanding of the pathogenesis of AD (Squitti et al., 2005). It should be emphasized that copper-related oxidative stress is also associated with states of copper deficiency, indicating that the fine regulation of copper concentrations within margins is critical. From the genetic standpoint, some loci in the ATP7B gene are associated with an increased risk of developing AD and Parkinson's disease (PD) (Squitti and Polimanti, 2013). ATP7B deactivation variants in transmembrane domains increase disease risk. Patients with some genetic background might be at greater risk of AD in the case of chronic copper exposure (Manto, 2014).

9-3 Parkinson's disease (PD)

Parkinson's is the second most common neurodegenerative disease (Manto, 2014). The pathological hallmark of the disease is related to the loss of dopamine-producing neurons

in the substantia nigra (pars compacta) (Manto, 2014). Alpha-synuclein is a copper-binding protein (with 2 sites for binding) enriched at the presynaptic terminals of neurons and catabolized by the ubiquitin-proteasome pathway (Rasia et al., 2005). Bound to copper, alpha-synuclein promotes the contribution of iron in biosynthesis of free radicals by exerting a ferrireductase effect (Manto, 2014). Triplication of the gene encoding the protein causes a familial PD (Singleton et al., 2003). Copper might be missing in neurons of the striatum, being no longer available for the CuZn-SOD, which is a major contributor of the antioxidant system in striatal neurons; in addition, decreased copper in striatal neurons would result in iron accumulation (Manto, 2014).

9-4 Ceruloplasmin deficiency

Ceruloplasmin is a protein containing copper; it acts as an iron oxidase. Loss of CP ferroxidase activity leads to iron accumulation in the pancreas, liver and brain; it belongs to a group of neurodegenerative disorders with brain iron accumulation (NBIA) (Manto, 2014). It may be confused with WD due to very low or absent serum CP (protein) levels in patients who may present with symptoms and brain imaging findings suggestive of WD (Becaria et al., 2006).

HAWTHORN

1- Plant description

Hawthorn, a common name of all plant species in the genus Crataegus, is a thorny shrub or small tree that normally has bright green leaves, white flowers, and dark red berries, each containing one to three or five seeds (Heinerman, 1996). It is often present only as a hedge plant in rural districts but it may spread to adjacent waste lands, riverbeds, forest remnants and hill country farmland (Healy, 1969). The two most common species used are *Crataegus laevigata* (syn *Crataegus oxyacantha*) and *Crataegus monogyna* (ShalizarJalali, et al., 2011). More than 20 species of hawthorn are used as herbal drugs in the world (Chang, 1986) where some of them are officially listed in the pharmacopoeias of many countries such as China, Germany, France, and England (Chang et al., 2002). The extracts of *Crataegus* are rich in proanthocyanidins and flavonoids (Ljubuncic et al., 2005). Many of these phenolic compounds have been shown to be cytoprotective by scavenging superoxide anion, hydroxyl radical, hydrogen peroxides and reducing lipid peroxidation (Rice-Evans, 2004). Hawthorn uses have included the treatment of digestive ailments, dyspnea, kidney stones and cardiovascular disorders. Today, hawthorn is used primarily for various cardiovascular ailments (Altinterim, 2012).

2- Historical use

Hawthorn has a long history of use in traditional Chinese medicine (TCM) and European herbal medicine. In Europe, the use of hawthorn can be dated back to the time of Discords in the first century A.D (Tyler, 1993). Its use for the treatment of heart disease began in the late 1800s (McCaleb et al., 2000). Hawthorn fruit is consumed not only for medicinal purposes mentioned above but also as food stuff such as canned fruit, jam, jelly, and drink (Chang et al., 2002).

3- Medicinal use

Hawthorn used to treat some human diseases (Kim et al., 2000), cardiac diseases, hypertension, hyperlipidemia, and as anti-atherosclerotic agent (Chang et al., 2005), myocardial injuries, angina pectoris, arrhythmia (Antsyshkina et al., 1990). In addition, it has been used for improving blood circulation, as well as blood stasis elimination (Chang et al., 2005). Hawthorn has also been used for the treatment of gastrointestinal diseases, stimulation of digestion, and promotion of stomach functions. Moreover, hawthorn had application in the treatment of indigestion, epigastric distension, abdominal pain, and diarrhea. In the European tradition, hawthorn is also used as an anti-spasmodic, cardiotonic, astringent, and diuretic agent (Edwards et al., 2012). Also hawthorn uses to treat hypercholesterolemia by decreasing LDLs and triglycerides (Nabavi et al., 2015). North African people use the hawthorn to treat sexual weakness (Dupont et al., 2011). It is found that hawthorn can treat nervous system disorders, anxiety and depression by decreasing ROS produced in the brain (Zhang et al., 2004).

4- Hawthorn chemical composition

Hawthorn fruits, leaves, and flowers contain a number of chemical constituents, which make it a powerful herb applied against many diseases. *C. monogyna* contains an important amount of polyphenols in different parts of a the plant (Simirgiotis, 2013). Also, flavonoids are present in C. monogyna (Bouzid et al. 2011). Phenolic content of the plant is influenced mainly by genetic factors (Kostić et al., 2012). Extrinsic factors may also influence phenylpropanoid metabolism (kostić et al., 2012), as altitude, temperature, light, soil nutrient content, etc. and intensity of light (Atanasova and Ribarova, 2009). Flavonoids are a class of polyphenolic compounds, due to their numerous pharmacological activities, ranging from antioxidant capacity to protective activity against chronic disease, such as cancer, diabetes, and inflammation (Habtemariam et al., 2014). Flavonoids are subdivided into several classes, such as flavones, flavonols, flavanones, flavans, anthocyanidins, isoflavones, neoflavones, and

chalcones (Nabavi et al., 2015), Procyanidins (Bahorun et al., 2003), Anthocyanin and Anthocyanidins (responsible for the *C. monogyna* color) (Nabavi et al., 2015) and Triterpenes (Caligiani et al., 2013) which all are present in the hawthorn.

Moreover, many oils and vitamins are present in the *C. monogyna* as linoleic acid, oleic acid, oxalic acid bis (trimethylsilyl) ester, palmitic acid and tetracosamethylcyclododecasiloxane, in addition to vitamins such as vitamin C (Rosario et al., 2013) and vitamin E (Keser et al., 2015) ...etc.

5- Hawthorn antioxidant activity

The main biologically active substances detected in the raw material of monogynous hawthorn are flavonoids and their glycosides: hyperoside (quercetin-3-galacto- side), quercetin, vitexin, vitexin-O-rhamnoside, isovi- texin-O-rhamnoside, acetylvitexin-O-rhamnoside, rutin, quercitrin (quercetin-3-rhamnoside), orientin, kaempferol, spireoside, saponaretin, oligomeric pro-cyanidins, catechins, and phenolic acids (chlorogenic acid, caffeic acid, triterpene saponins, …etc.) (Cui et al., 2006; Bahorun et al., 2003). Active compounds possess different free radicals scavenging ability (Masteikova et al., 2007). It can normalize the levels of antioxidant enzymes in the liver, aorta, and heart (Shanthi et al., 1996) and increase the activity of superoxide dismutase (Dai et al., 1987), scavenging free radicals (Bernatoniene et al., 2008) and straighten the antioxidant system and metal chelating activity (Keser et al., 2014), while certain flavonoids in some plant foods that inhibit copper uptake (Kuo et al., 1998).

6- Hawthorn side effect

No significant adverse events have been reported in clinical trials. The acute toxicity (LD50) is 18.5 ml/kg in mice and 33.8ml/kg in rats by oral administration of a 10% alcoholic extract solution of hawthorn leaves and fruits (Hiyama and Tosaka, 1969). Hawthorn may have a potentiating effect on digitalis, beta-blockers, and other hypotensive drugs. This

potential drug interaction may be related to the cardiotonic and hypotensive effects of hawthorn (Chang et al., 2002).

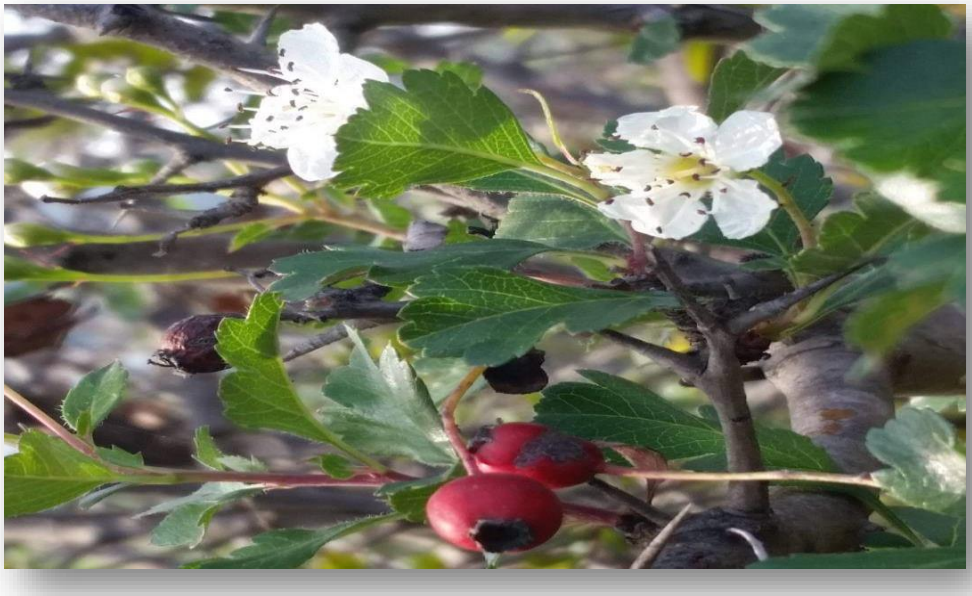

Figure 5: The Hawthorn *Crateagus monogyna* Jacq (personal photo).

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CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

1- Biological material:

The Wistar rats used in this experiment were purchased from Paster institute (Algiers). Adult rats are housed in a pet center of the University of Badji Mokhtar Annaba under natural photoperiodic and temperature conditions. Males weighed between 230g to 237g Rats had supplied with tap water and standard diet obtained from ONAB (Bejaia).

2- Chemicals:

Copper sulfate $CuSO_4$ is a blue color odorless crystalline substance soluble in water. Copper (II) sulfate is the inorganic compounds with the chemical formula $CuSO_4(H2O)x$, where x can range from 0 to 5. In this study, 100mg $CuSO_4(H2O)_5$/kg bw was used.

3- Plant preparation:

The hawthorn *Crataegus monogyna* was collected from the region of Sidi Amar, Annaba (north-east Algeria) in September, where fruits and leaves were cleaned by distilled water. 1.5 g of fresh fruits and leaves was crashed separately and 40ml of distilled water was added and kept overnight (12hours) to obtain a red and green solutions, respectively. The two solutions were immediately filtered and were administrated to rats daily in the morning by gavage during 30 consecutive days.

4- Experiment design

The study was carried out using Thirty six male Wistar rats, divided into 6 groups; the control group, the copper group (Cu) received 100 mg/kg bw, the fruits group (F) received 1.5 g/kg bw, the leaves group (L) treated with 1.5 g/kg bw; other two groups treated with the combination of Cu-L and Cu-F with the same doses.

After 30 days of treatment Wistar rats were sacrificed by decapitation, the blood was collected in heparinized, dry and EDTA tubes. After that, heparinized and dry tubes were immediately

centrifuged at 4000 rpm/5min, in which plasma and serum were obtained. Liver, kidney, and testis were weighed and then stored at -20 °C.

Table 1. The table of the experimental design procedure.

Control	Male receive tap water and standard diet *ad libitium* for 30days.
Cu group	Rats received 100mg/kg bw of copper sulphate with tap water and standard diet (1ml to each rat by gavage for 30 days).
F group	1.5 g/kg bw of fruits aqueous extract was given to rats (1ml to each rat by gavage for 30 days).
L group	1.5 g/kg bw of leaves aqueous extract was given to rats by gavage (1ml to each rat by gavage for 30 days).
CuF group	Rats received the mixture of 1.5g/kg bw of fruits with 100mg/kg bw of copper sulphate (1ml to each rat by gavage for 30 days).
CuL group	Rats received the mixture of 1.5 g/kg bw of leaves with 100mg/kg bw of copper sulphate (1ml to each rat by gavage for 30 days).

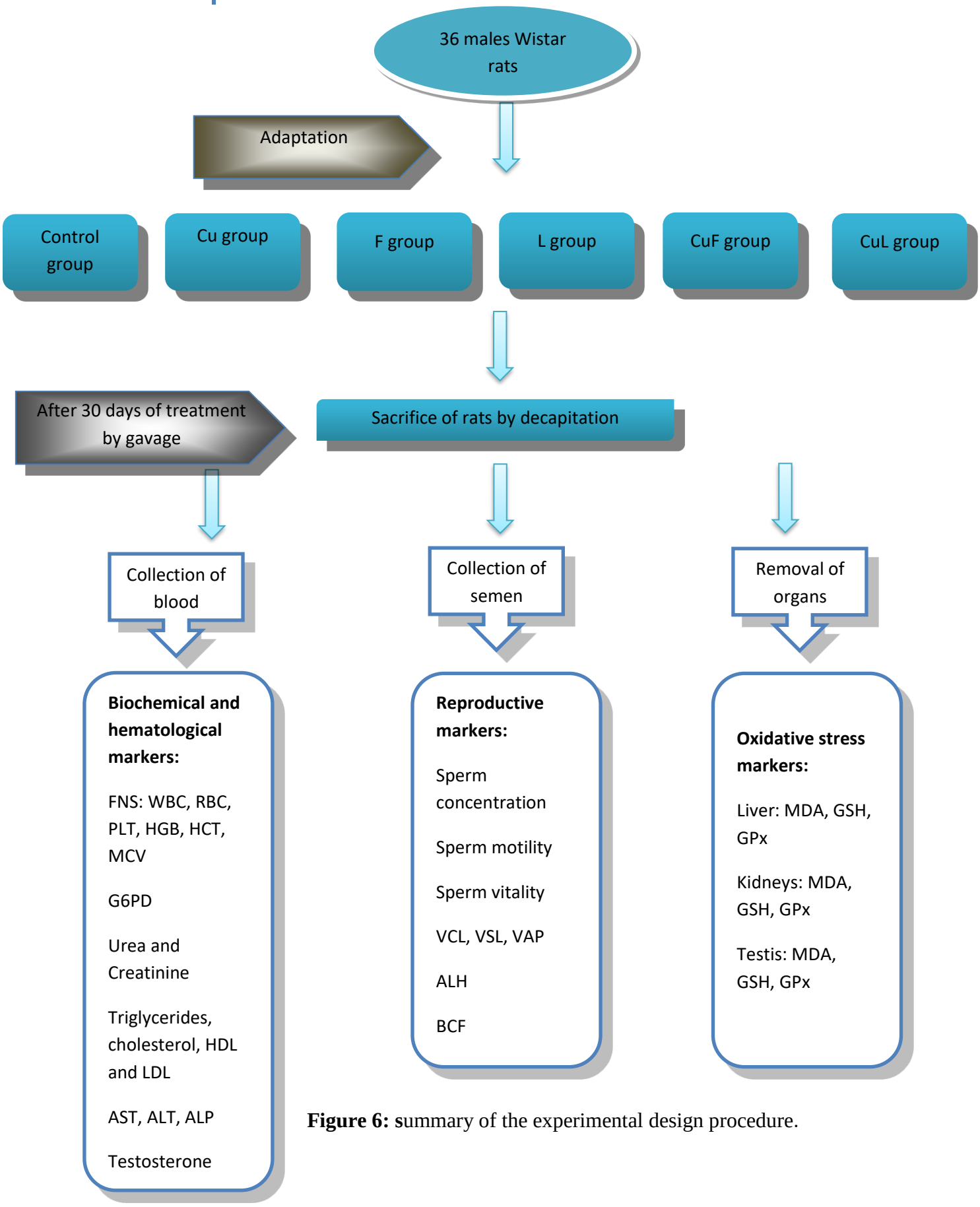
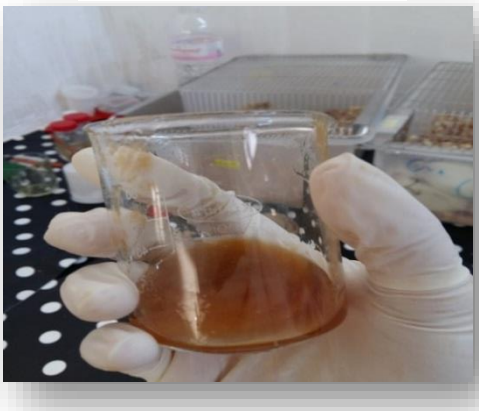


Figure 6: summary of the experimental design procedure.

Fruits

Leaves

Fruits aqueous extract



Leaves aqueous extract

Copper sulfate

Copper sulfate extract

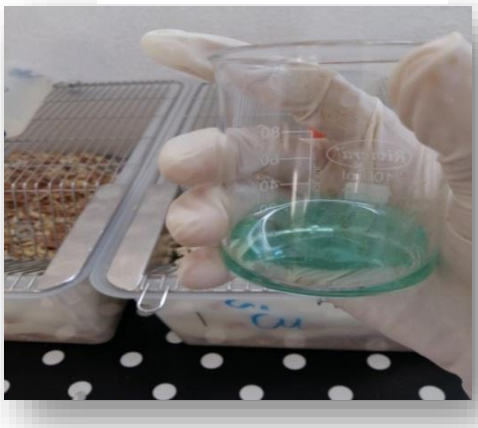


Figure 7: preparation of the hawthorn leaves and fruits aqueous extract and the copper solution.

5- Reproductive parameters:

The semen analysis has been realized using the (Comuter-Assisted Sperm Analysis Method (CASA) using Sperm Class Analysis (SCA® Microptic, Barcelona, Espagne).

5-1 Sperm analysis:

The Epididymis semen was obtained immediately after sacrifice, and then a drop of semen (about 1µl) was diluted with a physiological solution of NaCl 0.09% (1:8), and 5µL of the mixture was placed in an empty chamber slide (GoldCyto model). The slide was then placed on a Nikon Eclipse (Nikon E200-LED) microscope at the objective phase (x4). The sperm markers of concentration, motility, vitality, and velocity (VCL, VSL and VAP), the amplitude of lateral head displacement (ALH) and the beat cross frequency (BCF) were calculated.

5-2 Hypo-osmotic swelling test:

Hypo osmotic swelling test (HOST) had evaluated the integrity of sperm by exposing a drop of sperm derived from the epididymis cauda to the hypo osmotic solution (fructose, sodium citrate (jeyendran, et al., 1984). Then, using the objective of 40X, 200 sperm was counted, where the live sperm had showed a swelling of the tail.

5-3- Testosterone:

Testosterone was estimated using enzyme-linked immunosorbent assay technique (ELISA) by the ELISA apparatus (Columbus Pro TECAN), while results were read using (TECAN Sunrise).

The Ultrasensitive TESTOSTERON ELISA test (DRG instrument GnbH) is based on the principle of a solid phase enzyme-linked immunosorbent assay. The assay system utilizes a unique monoclonal antibody directed against a district antigenic determinant on the intact Testosterone molecule. Mouse monoclonal anti-testosterone antibody is used for solid phase (microtiter wells) immobilization, and goat anti-Testosterone antibody is used in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample is allowed to react

simultaneously with the antibodies. After 2 hours incubation at room temperature with shaking, the solid phase and enzyme was ashed with distilled water to remove unbound labeled antibodies. A solution of tetramethylbenzidine (TMB) was added and incubated for 20 minutes, resulting in the development of blue color. The color development is stopped with the addition of 1N HCl, and the formed yellow color was measured at 450 nm. The concentration of testosterone is directly proportional to the color intensity of the test sample.

6- Biochemical markers:

Biochemical parameters were measured by automated apparatus (Diatron PICTUS 200).

6-1 Urea:

The dosage of urea has been carried out by the colorimetric enzymatic method according to the technical data sheet of the Spinreact Kit (Spain).

Principle:

Urea in the sample is hydrolyzed enzymatically into ammonia (NH4+) and carbon dioxide (CO2). Ammonia ions formed reacts with salicylate and hypochlorite (NaClO), in presence of the catalyst nitroprusside, to form a green indophenol:

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} (NH_4)_2 + CO_2$$

$$NH_4 + \text{Salicylate} + \text{NaCl} \xrightarrow{\text{Nitroprusside}} \text{Indophenol}$$

The intensity of the color formed is proportional to the urea concentration in the sample (Kaplan, 1984).

6-2 Creatinine:

The dosage of creatinine has been carried out by the enzymatic method colorimetric according to the technical data sheet of the Spinreact Kit (Spain).

Principle:

The assay is based on the reaction of creatinine with sodium picrate as described by Jaffé.

Creatinine reacts with alkaline picrate forming a red complex. The time interval chosen for measurements avoids interferences from other serum constituents.

The intensity of the color formed is proportional to the creatinine concentration in the sample (Murray, 1984).

6-3 Activity of aspartate aminotransferase:

The dosage of ASAT (GOT) has been carried out by the colorimetric enzymatic method according to the technical data sheet of the Spinreact Kit (Spain).

Principle:

Aspartate amino transferase (AST), initially known as oxaloacetic glutamate transaminase (GOT), catalyzes the reversible transfer of an aspartate moiety to alpha-ketoglutarate to form glutamate and oxalacetate. The product oxaloacetate is reduced to malate in the presence of dehydrogenated (MDH) and NADH:

$$\text{Aspartate} + \alpha\text{-Ketoglutarate} \xrightarrow{\text{AST}} \text{Glutamate} + \text{Oxaloacetate}$$

$$\text{Oxaloacetate} + \text{NADH} + H^{+} \xrightarrow{\text{MDH}} \text{Malate} + NAD^{+}$$

The rate of reduction of the concentration of NADH in the center, determined numerically, is proportional to the catalytic concentration of AST in the sample (Murray, 1984).

6-4 Activity of alanine aminotransferase:

The dosage of ALAT (GPT) has been carried out by the enzymatic method according to the technical data sheet of the Spinreact Kit (Spain).

Principle:

Alanine aminotransferase (ALT) o Glutamate pyruvate transaminase (GPT) catalyses the reversible transfer of an amino group from alanine to α-ketoglutarate forming glutamate and

piruvate. The pyruvate produced is reduced to lactate by lactate dehydrogenase (LDH) and NADH:

$$\text{Alanine} + \alpha\text{-Ketoglutarate} \xrightarrow{\text{ALT}} \text{glutamate} + \text{pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + H^{+} \xrightarrow{\text{LDH}} \text{lactate} + NAD^{+}$$

The rate of decrease in concentration of NADH, measured photometrically, is proportional to the catalytic concentration of ALT present in the sample (Murray, 1984).

6-5 Activity of alkaline phosphate:

The dosage of alkaline phosphatase (ALP) has been carried out by the colorimetric enzymatic method according to the technical data sheet of the Spinreact Kit (Spain).

Principle

Photometric test according to the International Federation of Clinical Chemistry and Laboratory Medicines (IFCC), alkaline phosphatase (ALP) catalyzes the transfer of the phosphate group from p-nitrophenylphosphate (pNPP) to 2-amino-methyl-1 propanol by releasing p-nitrophenol and phosphate:

$$\text{p-Nitrophenylphosphate} + \text{AMP} \xrightarrow{\text{PAL}} \text{p-Nitrophenol} + \text{Phosphate}$$

The rate of formation of p-nitrophenol, determined so photometric is proportional to the catalytic concentration of alkaline phosphatase in the tested sample (Wenger *et al.*,1984; Rosalki*et al*., 1993).

6-6 Determination of triglycerides:

Triglyceride has been measured using the colorimetric enzymatic method according to the technical user manual of the Spinreact Kit (Spain).

Principle:

The triglyceride incubated with lipoprotein lipase (LPL), release the glycerol and free fatty acids. Glycerol is converted to glycerol-3-phosphat (G3P) and adenosine-5-diphosphate (ADP) by the glycerol kinase and adenosine phosphate (ATP). The glycerol-3-phophate (G3P) is then converted by the glycerol phosphate dehydrogenase (GPO) in active ingredient dihydroxyacetone phosphate (DAP) and hydrogen peroxidase (H_2O_2). In the last reaction, the hydrogen peroxide (H_2O_2) reacts with 4-aminophenazone (4-AP) and p-chlorophenol in the presence of peroxidase (POD) to give a red color.

The protocol therefore includes the following reactions:

$$\text{Triglyceride} + H_20 \xrightarrow{\text{LPL}} \text{Glycerol + Free Fatty Acids}$$

$$\text{Glycerol + ATP} \xrightarrow{\text{Glycerolkinase}} \text{G3P + AD}$$

$$\text{G3P} + O_2 \xrightarrow{\text{GPO}} \text{DAP} + H_2O_2$$

$$H_2O_2 + \text{4-AP + P-chlorophenol} \xrightarrow{\text{POD}} \text{Quinone} + H_2O_2$$

The intensity of the red color is proportional to the concentration of triglycerides in the sample (Buccolo and David 1973).

6-7 determination of cholesterol:

The dosage of high density lipoproteins HDL has been carried out by the colorimetric enzymatic method according to the technical data sheet of the Spinreact Kit (Spain).

The cholesterol present in the sample gives rise to a colored compound, following the following reaction:

$$\text{Ester cholesterol} + H_20 \xrightarrow{\text{CHE}} \text{cholesterol + fatty acids}$$

$$\text{Cholesterol} + O2 \xrightarrow{\text{CHOD}} \text{4-cholesteronone} + H_20_2$$

$$2H_2O_2 + \text{Phenol + 4-aminophenazone} \xrightarrow{\text{POD}} \text{Quinonimine} + 4H_2O$$

The intensity of the color formed is proportional to the concentration of cholesterol present in the tested sample (Naito, 1984 ; Meiattini*et al*., 1978).

6-8 Determination of high density lipoproteins:

The dosage of high density lipoproteins-cholesterol (HDL) has been carried out by the colorimetric enzymatic method according to the technical data sheet of the Spinreact Kit (Spain).

Principle:

The very low density (VLDL) and low density (LDL) lipoproteins from serum or plasma are precipitated by phosphotungstateinthe presenceof magnesium ions. After centrifugation the supernatant contains high density lipoproteins (HDL). The HDL cholesterol fraction is determined using the total cholesterol enzymatic reagent (Naito, 1984; Grove, 1979).

6-9 Determination of low density lipoproteins:

The dosage of low density lipoproteins-cholesterol (LDL) has been carried out by the colorimetric enzymatic method colorimetric according to the technical data sheet of the Spinreact Kit (Spain).

Principle:

Directly determination of serum LDLc (low-density lipoprotein cholesterol) levels without the need for any pre-treatment or centrifugation of the sample (Weiland and Seidel, 1983; Friedewald*et al*., 1972).

The assay takes place in two steps.

Elimination of lipoprotein no-LDL:

$$\text{Cholesterol esters} \xrightarrow{\text{CHE}} \text{Cholesterol + Fatty acids}$$

$$\text{Cholesterol + } O_2 \xrightarrow{\text{CHOD}} \text{4-cholestenone + } H_20_2$$

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

Measurement of LDLc:

$$\text{Cholesterol esters} \xrightarrow{\text{CHE}} \text{Cholesterol + Fatty acids}$$

$$\text{Cholesterol + } O_2 \xrightarrow{\text{CHOD}} \text{4-cholestenone + } H_20_2$$

$$2H2O2 + TODS + 4\text{-}AA \xrightarrow{\text{POD}} \text{Quinonimine + } 4H_2O$$

The intensity of the color formed is proportional to the LDLc concentration in the sample.

7- Oxidative stress markers:

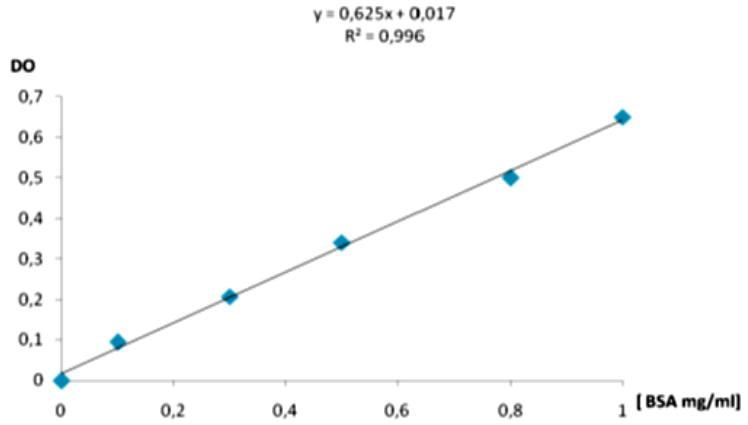
7-1 Measurement of proteins:

Principle: Proteins' concentration was determined by the method of Bradford (1976) using Coomassie Blue (G 250) as a reagent. The amino groups (-NH 2) of the proteins react with a reagent based on orthophosphoric acid, ethanol and Coomassie blue to form a blue complex. The appearance of this color reflects the degree of ionization of the acid medium and the intensity establishes the concentration of proteins in the sample.

Operating mode

1. Take 0.1 ml of the homogenate.
2. Add 5 ml of Bradford's reagent.
3. Shake and allow standing for 5 minutes for color stabilization.
4. Read the optical density at 595 nm, against the white.
5. The optical density obtained is reported on a calibration curve previously drawn.

The concentration of the proteins is determined by comparison with a standard range of bovine serum albumin (1 mg / ml) carried out under the same conditions.



7-2 Measurement of malondialdehyde:

Principle: Malondialdehyde (MDA) is a product of the lipid peroxidation reactions that are formed during the attack of polyunsaturated lipids by reactive oxygen species generated by certain contaminants. In our study, testis, hepatic and renal MDA levels were evaluated according to the method of Ohkawa*et al* (1979)**.** The assay is based on the formation in hot and acidic medium (100 ° C.) between MDA and thiobarbituric acid (TBA) of a colored pigment absorbing at 530 nm, extractable by organic solvents such as butanol.

Preparation of the homogenate:

500 mg of liver or kidney of the different groups are ground cold with the aid of an ultrasound homogenizer in the presence of 5 ml of a phosphate buffer solution (0.1 M, pH 7.4) to obtain a homogenate.

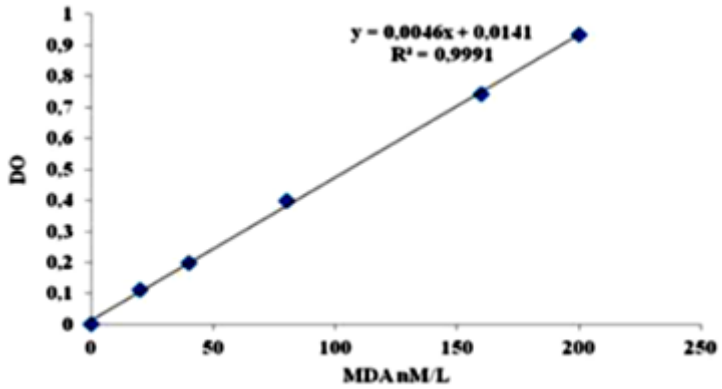
Operating mode:

- Take 0.5 ml of the homogenate.
- Add 0.5 ml of trichloroacetic acid (TCA) 20%.
- Add 1 ml of thiobarbituric acid (TBA) 0.67%.
- Mix and incubate in a water bath at a temperature of 100 ° C for 15 minutes.
- Cool and add 4 ml of n-butanol.
- Centrifuge for 15 minutes at 3000 rpm.

Recover the supernatant, and read the optical density at 530 nm against the standard.

Calculation of MDA concentration:

The amount of MDA in the sample is expressed in nmol / gram of tissue (liver, kidneys and testis). It is obtained from the standard curve made with 1, 1', 3,3'-tetraethoxypropane made under the same conditions.



7-3 Measurement of glutathione:

Principle: Glutathione (GSH) assay was performed according to the method of Wekbeker and Cory (1988)**.** The principle of this assay is based on the measurement of the optical absorbance of 2-nitro-5-mercapturic acid. This last results from the reduction of 5, 5'-dithio-bis-2-nitrobenzoic acid **(**Ellman's reagent, DTNB) by the (-SH) groups of glutathione. For this, a deproteinization of the homogenate is essential in order to keep only the specific thiol groups of glutathione.

Preparation of the homogenate:

200 mg of tissue (liver, kidneys and testis) were placed in the presence of 8 mL of 0.02 M Ethylene Diamine Tetra Acetic Acid (EDTA) solution and then cold milled using an ultrasonic homogenizer to obtain a homogenate.

Operating mode:

- Take 0.8 ml of the homogenate.
- Deproteinize by adding 0.2 ml of a 0.25% sulfosalicylic acid solution.
- Stir the mixture and leave for 15 minutes in an ice bath.

- Centrifuge at 1000 rpm for 5 min.
- Take 0.5 ml of the supernatant.
- Add 1 ml of Tris + EDTA buffer (0.02 M EDTA), pH 9.6.
- Mix and add 0.025 ml of 0.01 M DTNB (dissolved in absolute methanol).
- Leave for 5 min at room temperature for color stabilization that develops instantly.
- Read the optical densities at 412 nm against the standard.

Calculation: the concentration of glutathione is obtained by the following formula.

$$[GSH](nM\ GSH/mg\ protide) = (DO \times 1 \times 1.525)/(13100 \times 0.8 \times 0.5 \times mg\ proteins\ )$$

OD: Optical density at 412 nm.

1: Total volume of solutions used in deproteinization (0.8 ml homogenate + 0.2 ml SSA).

1.525: Total volume of the solutions used in the GSH assay (0.5 ml supernatant + 1 ml Tris EDTA + 0.025 ml DTNB).

13100: Absorbance coefficient of the group (-SH) at 412 nm.

0.5: Volume of the supernatant found in 1.525 ml.

0.8: Volume of the homogenate found in 1 ml.

It is noted that the concentration of GSH is measured by contribution to 1 mg of protein. This is why this dosage must be accompanied by the protein assay.

7-4 Activity of gluthathione peroxidase:

Principle: The enzymatic activity of glutathione peroxidase (GPx) was measured by the method of Flohe and Gunzler (1984)**.** This method is based on the reduction of hydrogen peroxide (H 2 O 2) in the presence of reduced glutathione (GSH), the latter is transformed into (GSSG) under the influence of GPx according to the following reaction:

$$H_2O_2 + 2GSH \longrightarrow GSSG + 2H_2O$$

Preparation of the homogenate:

500 mg of liver, kidney and testis of the different groups are cold milled using an ultrasound homogenizer in the presence of 5 ml of a solution of TBS (50 mMTris, 150 mMNaCl, pH 7.4) to obtain a homogenate.

Operating mode:

- Take 0.2 ml of the homogenate.
- Add 0.4 ml of GSH (0.1 mM).
- Add 0.2 ml of the TBS buffer solution (50 mMTris, 150 mMNaCl pH 7.4).
- Incubate in a water bath at 25 ° C for 5 min.
- Add 0.2 ml of H 2 O 2 (1.3 mM) to initiate the reaction, leave to act for 10 minutes.
- Add 1 ml of TCA (1%) to stop the reaction.
- Put the mixture in the ice for 30 minutes.
- Centrifuge for 10 minutes at 3000 rpm.
- Take 0.48 ml of the supernatant.
- Add 2.2 ml of the TBS buffer solution.
- Add 0.32 ml of DTNB (1.0 mM)
- Mix and after 5 minutes read the optical densities at 412 nm.

Calculation: the determination of the enzymatic activity of the GPx is done using the following formulas:

$$x = \frac{DO\ sample - DO\ standard\ \times 0{,}04}{DO\ standard}$$

$$\text{GPx (µmol /mg proteins)} = \frac{GSH\ quantity\ disapeared}{[\quad]ofproteins}$$

X: Quantity of reduced GSH disappeared (oxidized) in 0.2 ml extracted in 1 ml.

OD sample: Optical density of the sample.

Standard OD: Optical density of the standard.

0.04: Substrate concentration (GSH).

7-5- Glucose-6-phosphate dehydrogenase measurement:

Erythrocytes glucose-6-phosphate dehydrogenase (G6PD) was measured using Mindray BS-380. According to BIOLABO REAGENT (U.V Kinetic method) kit and the reaction scheme: method of Beutler et al., (1977), while the rate of increase in NADPH concentration measured at 340nm is proportional to the G6PD activity in the specimen.

Principle:

Reaction scheme (method of Beulter and al.) is as following:

$$\text{G-6-P+NADP+} \xrightarrow{\text{G-6-PDH}} \text{6-P-Gluconate + NADPH + } H^{+}$$

The rate of increase in NADPH concentration measured at 340nm is proportional to the G-6-PDH activity in the specimen.

Sample handling for determination of the G6PD activity in erythrocytes:

1 Homogenize the sample (whole blood) by successive slow reversals.

2 Up-and-down movements with the pipette plunger, remove 0.2ml of sample. Wipe the outside of the tip.

3 Transfer 0.2ml of sample in 2ml of NaCl (0.9 g/dl). Homogenise by up-and-down movements, and then by reversals of the tube (use a cap).

4 Centrifuge 3min at 1500 tpm.

5 Remove the supernatant taking care not to remove erythrocytes (let approximately 2mm of washing solution).

Introduce 2ml of NaCl (O.9 g/dl) in the tube. Mix gently until full resuspension of the residue.

6 Centrifuge 3min at 1500 tpm.

7 Repeat step 5, 6 and 7 to perform a third wash.

8 Remove a maximum of supernatant, being careful not to aspirate residue. Use a 200µl pipette to remove the last drops of washing solution.

9 Suspend the washed erythrocytes in 0.9 ml of haemolysing solution (vial R3). Place 15 min at 2-8°C and centrifuge 3 min at 1500 tpm. Use the supernatant (hemolysate) within one hour.

8- The complete blood count:

The complete blood count (CBC) or hemogramme was realized in the laboratory using the blood counter (Abacus 4).

9- Statistical analysis:

The measurement was realized using (MINITAB 18, ANOVA Tukey). All values are expressed as mean ± standard deviation. Means that do not share the same letter are significantly different ($p<0.05$), according to one-way ANOVA, followed by Tukey test.

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CHAPTER 3

REPRODUCTIVE PROFILE

RESULTS

1- Reproductive markers

1-1 Testosterone

Results showed after one month treatment that plasma testosterone concentration was significantly lower in the Cu group compared to the untreated control. When compared to the Cu group, testosterone was significantly higher in the CuF, CuL, CuF and CuL groups (Figure 8).

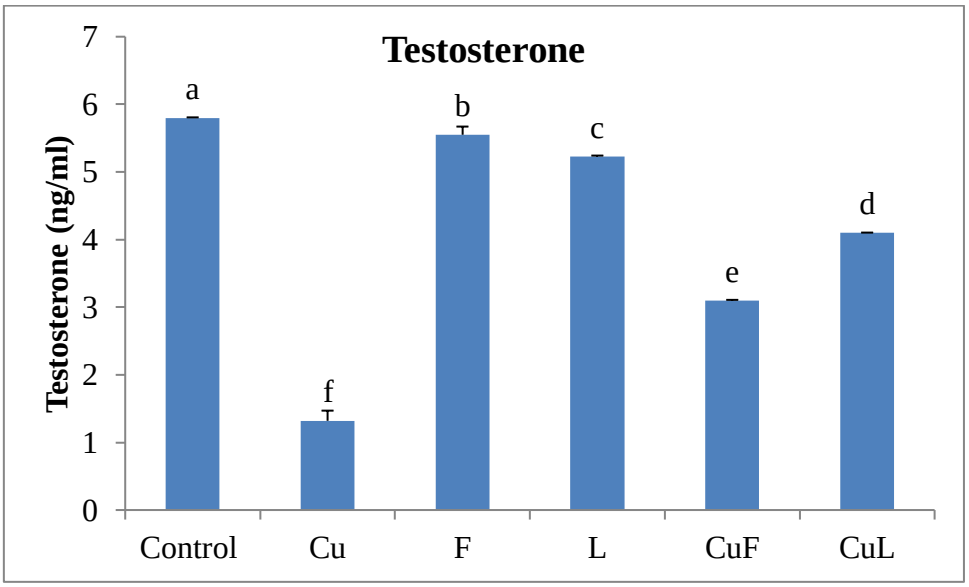

Figure 8: the level of testosterone in the groups treated by copper, hawthorn (F, L) and their combinations (CuF and CuL) for 30days. Means that do not share the same letter are significantly different at $p<0.05$.

1-2 Sperm concentration

Results of semen analysis indicated a significant lower sperm concentration in the Cu group compared to the control, however it showed no significant difference between the F group and the control, but it was significantly higher in the L group than in the control, and significantly higher in the CuF and CuL groups compared to the Cu group (Figure 9).

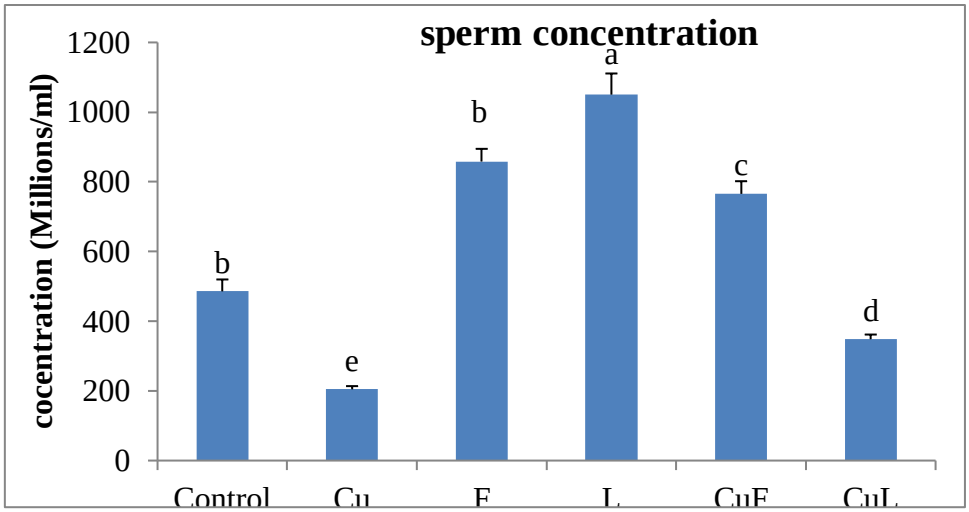

Figure 9: the sperm concentration in the groups treated by copper, hawthorn (F, L) and their combinations (CuF and CuL) for 30days. Means that do not share the same letter are significantly different at $p<0.05$.

1-3 Sperm motility

Sperm motility was significantly lower in both Cu group and the F group compared to the untreated control. Motility was also significantly lower in the CuF and CuL groups compared to the control, but higher than the Cu group (Figure 10).

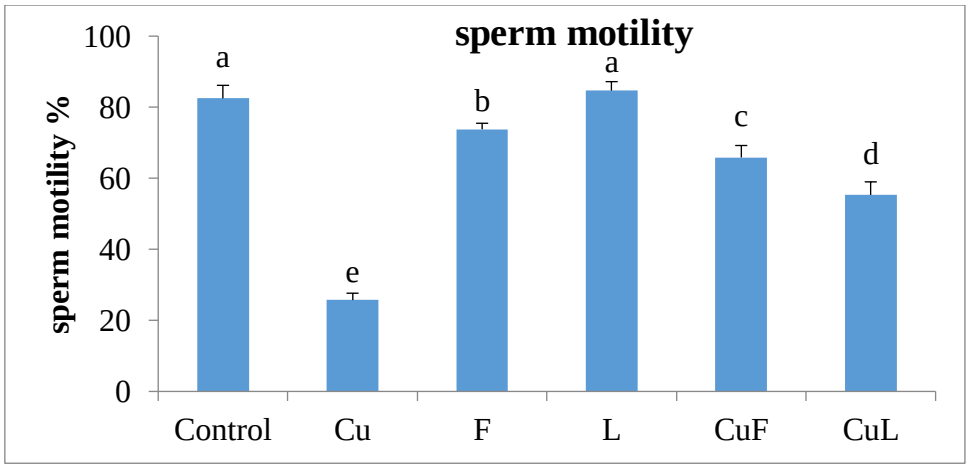

Figure 10: the sperm motility in the groups treated by copper, hawthorn (F, L) and their combinations (CuF and CuL) for 30days. Means that do not share the same letter are significantly different at $p<0.05$.

1-4 Sperm vitality

The percentage of dead sperm in the Cu group was significantly higher compared to the control, while F, L, CuF, and CuL groups showed levels close to that of the control (Figure). On the other hand, the percentage of dead sperm was remarkably lower in the combined treatments of CuF and CuL groups compared to the Cu exposed group. Live sperm of the Cu group was slightly decreased when compared to the control, with a weak reduction in the CuF and CuL groups, whereas F, CuF and CuL have kept a close values as that of the control (Figure 11).

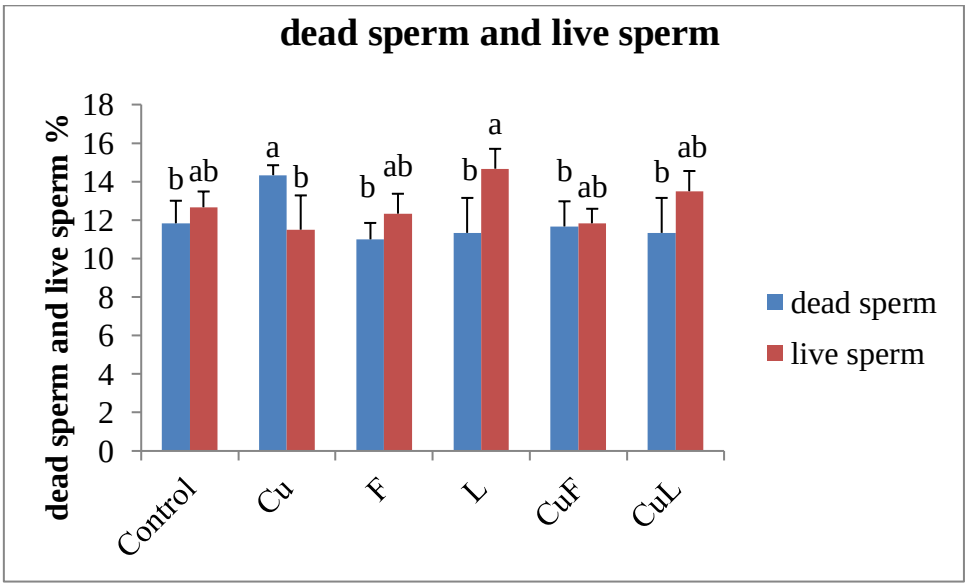

Figure 11: the dead and live sperm in the groups treated by copper, hawthorn (F, L) and their combinations (CuF and CuL) for 30days. Means that do not share the same letter are significantly different at $p<0.05$.

1-5 Sperm velocity

The VCL, VSL, and VAP of sperm from Cu group were significantly lower than that of the control. The VCL, VSL, and VAP of sperm in the CuF and CuL groups were significantly higher than that of the Cu group, and not statistically different from the control

group (Figure 12). The VCL and the VAP of the L positive control were significantly higher than that of the control group.

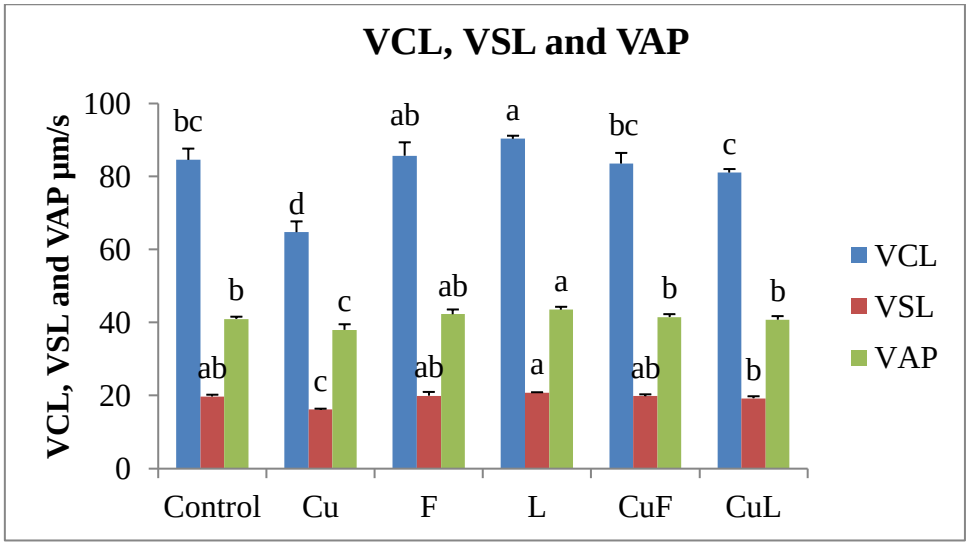

Figure 12: the velocity (VCL, VSL and VAP) in the groups treated by copper, hawthorn (F, L) and their combinations (CuF and CuL) for 30days. Means that do not share the same letter are significantly different at $p<0.05$.

1-6 Amplitude of lateral head displacement

The sperm ALH was significantly lower in the Cu group compared to the control. Sperm ALH in the other groups were not significantly different from that of the control (Figure 13).

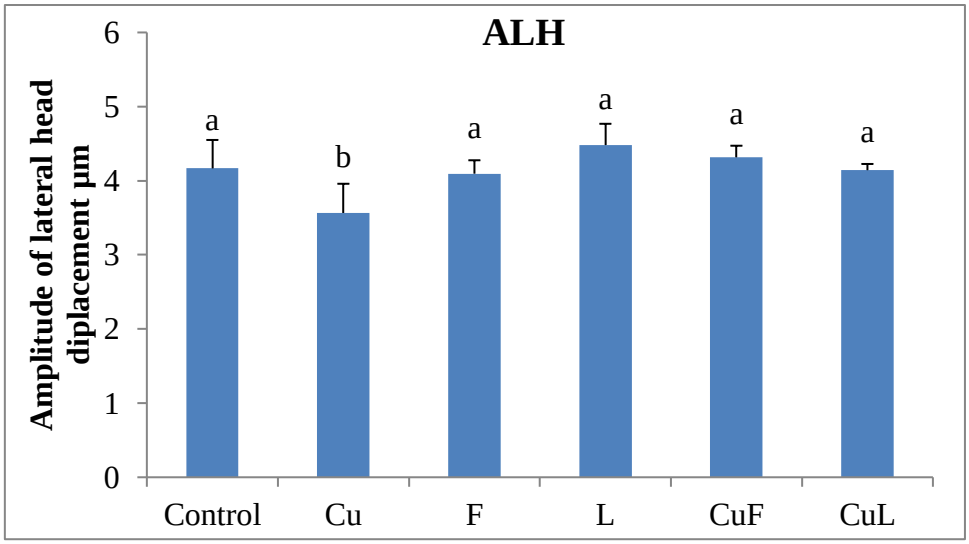

Figure 13: the amplitude of lateral head displacement in the groups treated by copper, hawthorn (F, L) and their combinations (CuF and CuL) for 30days. Means that do not share the same letter are significantly different at $p<0.05$.

1-7 Beat cross frequency

The BCF was lower in the Cu group compared to the control, while F, L, CuF and CuL groups did not differ significantly from that of the control (Figure 14).

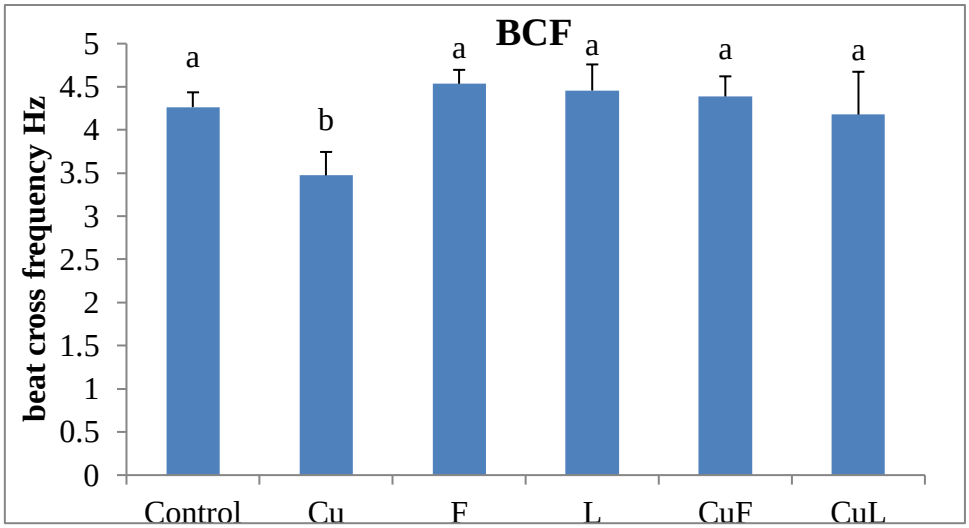

Figure 14: the beat cross frequency in the groups treated by copper, hawthorn (F, L) and their combinations (CuF and CuL) for 30days. Means that do not share the same letter are significantly different at $p<0.05$.

2- Oxidative stress

2-1 Malondialdehyde

The MDA level was increased significantly by Cu treatment compared to the control, but it decreased significantly in the CuF and CuL groups compared to the Cu group. A significant increase in the CuF, but CuL has kept the same level as that of the control (Figure 15).

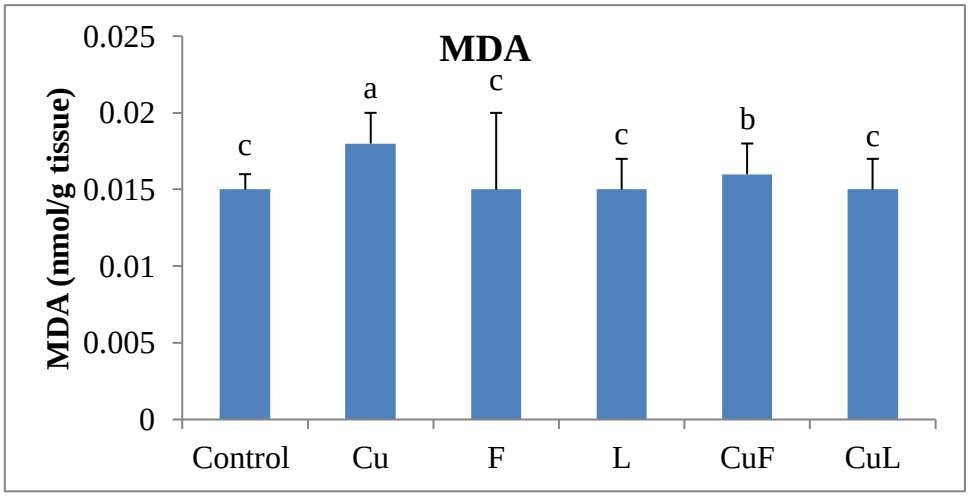

Figure 15: the MDA level in the groups treated by copper, hawthorn (F, L) and their combinations (CuF and CuL) for 30days. Means that do not share the same letter are significantly different at $p<0.05$.

2-2 Glutathione

Data of the GSH level and GPx activity has shown a significant drop in the Cu group compared to the control, albeit it increased significantly in the CuF and CuL groups compared to the Cu group and the control as well (Figure 16). There was no difference in the MDA level between the Cu group and those of the positive controls.

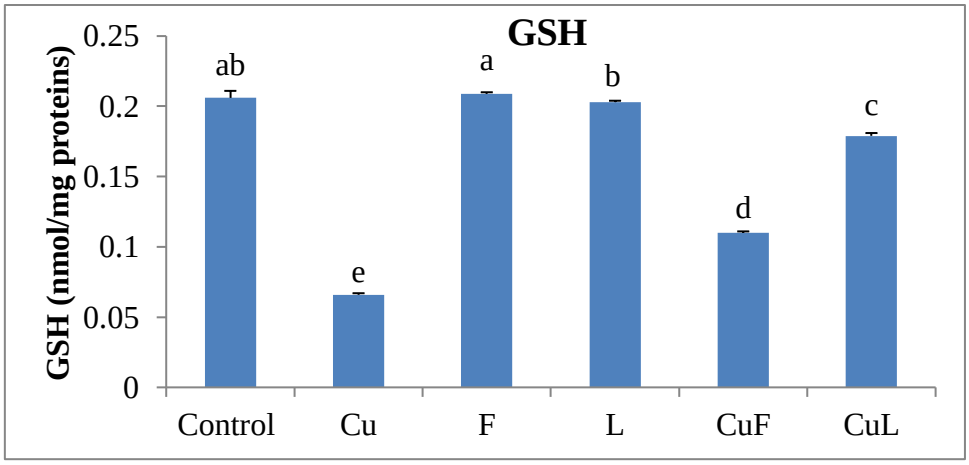

Figure 16: The GSH level in the groups treated by copper, hawthorn (F, L) and their combinations (CuF and CuL) for 30days. Means that do not share the same letter are significantly different at $p<0.05$.

2-3 Glutathione peroxidases

The GPx activity was lower in the Cu group compared to the control, although it increased significantly in the CuF and CuL groups compared to the Cu group and to the untreated control (Figure 17). The GPx activity of the Cu group was close to the positive controls.

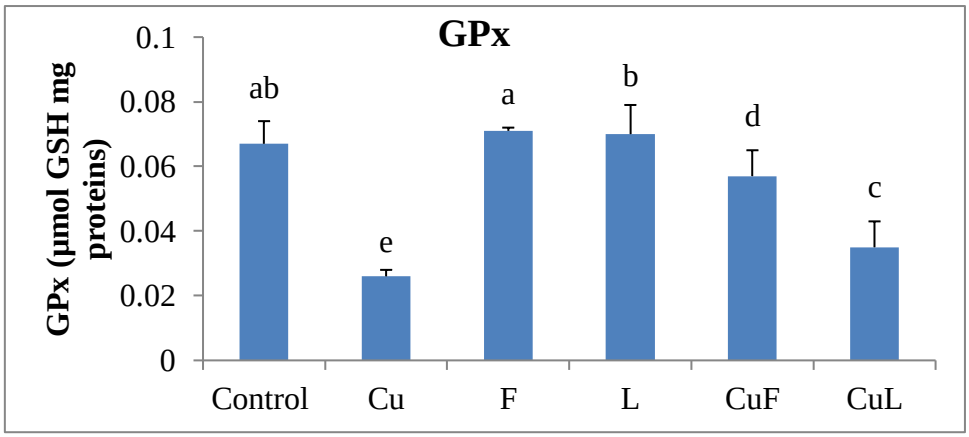

Figure 17: The GPx activity in the groups treated by copper, hawthorn and the mixture of copper, hawthorn (F, L) and their combinations (CuF and CuL) for 30days. Means that do not

share the same letter are significantly different at $p<0.05$.

DISCUSSION

Our finding showed a decline in the concentration, motility, VCL, VSL, VAP, ALH, BCR of sperm, in addition to the serum testosterone concentration in rats received copper sulphate during one month. Previous studies showed a correlation between the sperm quality and the presence of high Cu concentrations, in which high copper dose has lowered sperm motility (Roychoudhury et al., 2016) and sperm concentration (Roychoudhury et al. 2008). In fact many other studies showed that copper overdose in the seminal plasma has caused male infertility (Wong et al., 2001). Others showed that the administration of 100 and 150mg/kg/day of copper sulfate lowered significantly the sperm concentration and motility in mice administrated orally for 8 weeks (Chen et al., 2019).

Contrary to our results, $CuSO_4$ incubation with rabbit semen has increased the velocity and spermatozoa distance curved line after 2 hours of, but after 24h all spermatozoa seems to be died (Roychoudhury et al. 2010). Moreover, Eidi et al. (2009) reported a decrease in sperm concentration, vitality and motility of human who has high levels of semen copper, in addition to copper was found to lower the semen pH and spermatozoa concentration and motility. Other researchers found that the pH between 6.2 and 5.2 has led to a decrease in sperm concentration, movement, and velocity measures VCL, VSL and VAP (Zhouet al., 2015). Furthermore, the diminution of sperm concentration, vitality and motility perhaps was due to zinc deficiency due to the likelihood of antagonist between Cu and Zn (Kasperczyk et al., 2016). Previous investigations showed that the administration of 100 and 150 mg copper sulfate /kg/day for 8 weeks by oral gavage in mice may alter spermatogenesis by inducing apoptosis in testicular cells, while same finding showed no change in testosterone levels (Chen et al., 2019).

Copper as others heavy metals may disturb LH and FSH liberation by affecting the pituitary receptors (Chang et al., 2011). This may explains our results, as copper group showed a

significant decrease in testosterone concentration as LH and FSH are known to control the testosterone balance. Copper and zinc antagonism may lead to zinc deficiency, since the latter is involved in the formation of androgens (Bedwal and Bahuguna 1994). Testosterone level was decreased in rats when orally received 200mg CuSO4/kg bw for 30 days (Khushboo et al., 2017). Thus, Hammami et al. (2009) hypothesized that:"steroidogenesis can be inhibited in three different ways: (i) disorder in free cholesterol mobilization toward Leydig cell mitochondria, (ii) interruption of cholesterol mitochondria translocation with the steroidogenic acute regulatory (STAR) protein as an effector, and (iii) prevention of cholesterol conversion into testosterone by impairing activities of key regulatory enzymes of steroidogenesis". Possibly the reduction in serum testosterone level observed in this study may have been caused by one of these hypothesized mechanisms (Hammami et al. 2009).

Our investigations showed that copper had significantly increased the MDA and decreased the GSH and GPx. Copper ions may lead to lipid peroxidation, and free radicals generation may be explained by the augmentation of MDA.

Spermatozoa are more exposed to peroxidative damage, it contains high polyunsaturated fatty acids that induces ROS formation by the Fenton and Haber- Weiss reactions provoked by the copper toxicity (Huang et al., 2000); this may easily lead to its oxidative stress (Eidi et al., 2009). ROS are known to be one of the important provokers of male infertility by affecting the sperm chromatin condensation and regulate the count of germ cells by inducing apoptosis or the proliferation of spermatogonia (Agarwal et al., 2014), also it is very harmful to spermatozoa (Said et al., 2005) mainly by the destruction of sperm membrane (Agrawal et al., 2003) and can accelerate the potential of germ cells apoptosis (Agrawal et al., 2005).

GSH and GPx are free radicals scavengers; the augmentation of these markers was due to ROS generated by the testis cells induced by copper ions.

In Arabs countries the hawthorn is used to treat sexual weakness (Miller, 1998; Ju, 2005). As a powerful antioxidant, the hawthorn may protect Sertoli and leydig cells from the oxidative stress. Also the presence of many phenolic compounds, oils and vitamins was found in many studies that could possibly improve the sperm quality. As our results showed the improvement of the sperm quality in the positive group CuL and CuF, hawthorn certainly contains some antioxidants like vitamin C in both fruits and leaves.

Previous finding showed the presence of vitamin C in the hawthorn that proven its role on sperm quality (Rosario et al., 2013). Yousef et al (2003) have found that vitamin C was able to improve the sperm concentration in Awassi rams after 45 days of treatment with 50 and100 mg/kg bw of vitamin C by injection (Al-saab, 2015). Vitamin C seems to help in neutralizing hydroxyl, superoxide, and hydrogen peroxide radicals, which may prevent against sperm agglituation (Agarwal et al., 2004). Ascorbic acid in hawthorn (Rosario et al., 2013) can neutralize the odd electrons and the outer layer of the free radicals could be inhibited (Schuh, et al. 2004); it may block as well the copper intestinal absorption and help its excretion (Frieden E, 1986).

Our results showed an augmentation of sperm motility in the group treated with the leaves L as that reported by Hu &Xiong, (2006) who used hawthorn as medicinal additives by co-incubating its extracts with sperm of patient suffering from asthenospermia as medicinal additives. The presence of oils in *C. monogyna* (Bechkri et all 2017) could increase VSL and VAP. Additionally, the presence of vitamins, flavonoids, and essential oils in hawthorn (Alizadeh et al. 2015) might act as a source of energy, which can improve the movement of the spermatozoa, the ALH, BCR and velocity (VCL VSL) in rats of the combined treatments CuL and CuF.

Our results showed that MDA level was normally maintained in the combined groups CuL and CuF comparing to the copper group. Previously, the *C. monogyna* extracts was reported to act as an antioxidant (Froehlicher et al., 2009) by scavenging the free radicals and preventing the oxidation of LDL (Liu T et al., 2010) due to the presence of different components (Masteikova et al., 2007). Vitamin E in the hawthorn prevents against lipid peroxidation (Keser et al., 2015) by acting against chain-breaking, deactivate excited oxygen molecules, and scavenges peroxyl and alkoxyl radicals (Hwang and Dolores, 2013). Recently, linoleic acid (Omega-6), oleic acid (Omega-9), oxalic acid bis (trismethylsilyl) ester, palmitic acid and tetracosmethyl-cyclodecasiloxane from hawthorn have powerful antioxidants activity (Bechkri et al., 2017), which probably explain the decrease of MDA concentration and the increase of GSH level and GPx activity of the combined groups comparing to the Cu group. Moreover, omega 6 was confirmed to improve sperm motility (Putri et al., 2018), and Quercetin was already tested as growth inhibitor on human epididymal cancer (Kandaswami et al., 2005) and improving sex organs functions (Taepongsorat et al., 2008) by scavenging free radicals and chelating divalent cations (Seddiki et al., 2017). Such compound might have a role in reducing hydrogen peroxide, increasing sperm antioxidant defenses and preventing DNA damage induced by oxidative stress provoked by copper (Zribi et al., 2012). Catechines polyphenols of *C. monogyna* were able to reduce ROS by quenching free radicals and chelating transition metal (Nabavi et al., 2015), while catechines of the green tea were able to improve sperm quality and prevents against copper-induced toxicity (Sajid et al., 2018).

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CHAPTER 4

HEPATIC, NEPHROTIC AND LIPID PROFILE

RESULTS

1- Total body weights:

Results of the body weight has showed a regulated augmentation of weights in the control group during the 30 days of the experiment, while the group treated with Cu showed a diminution in the total body weight. Positive groups received *C. monogyna* extract showed a regulated increase in the body weight as that of the control, while CuF and CuL showed a slight decrease in the body weight (Table 2).

Table 2. Variation of total body weight (g) during the treatment of rats by Cu, F, L, CuF and CuL for 30 days.

	Control	Cu	F	L	CuF	CuL
Week 1	233.62	234.62	233.7	234.32	233	234.23
Week 2	240.61	233.2	232.76	232.11	230.32	230.43
Week 3	250.32	220.67	235.65	232.45	225.76	231.87
Week 4	258	210.32	240.87	239.87	222.87	230.54

2- Urea:

During the course of the present investigations, it was observed that the treatment of rats with Cu has caused a slight increase in urea comparing to the control (Figure 18), but it decreased slightly in the CuF group but CuL group has decreased significantly compared to the Cu group, while the positive groups treated with the *C. monogyna* extracts fruits F and leaves L have decreased but not considerably in urea comparing to the control.

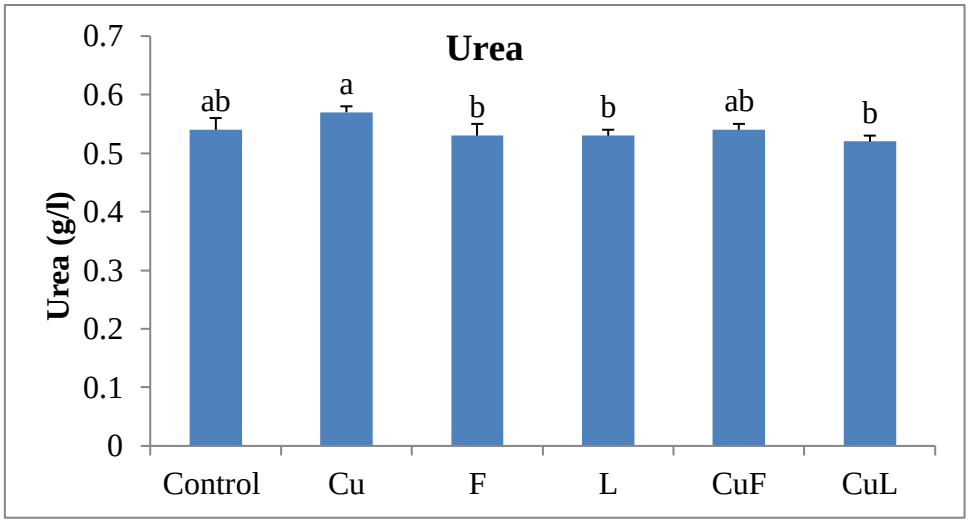

Figure 18: variation of urea level in the groups treated by Cu, F, L, CuF and CuL for 30 days. Means that do not share the same letter are significantly different at $p<0.05$.

3- Creatinine:

Results of creatinine showed that the treatment of rats with Cu has caused a significant increase compared to the control, but it decreased significantly in the CuL and CuF groups compared to the Cu group, while the positive groups F and L have preserved the same level of creatinine (Figure 19).

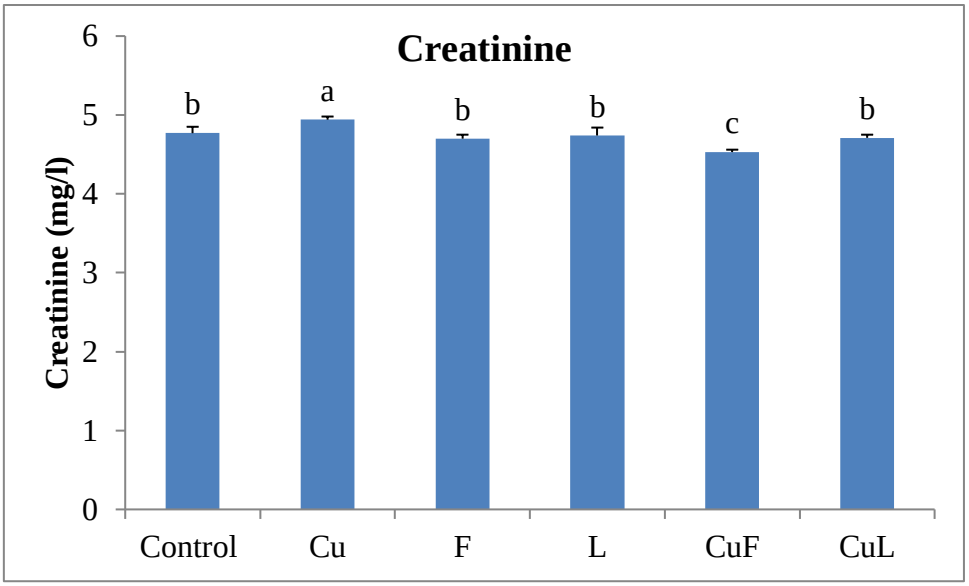

Figure 19: variation of creatinine level in the groups treated by Cu, F, L, CuF and CuL for 30 days. Means that do not share the same letter are significantly different at $p<0.05$.

4- ASAT, ALAT and ALP activity

Copper intake has significantly increased the ASAT and the ALAT activity compared to control, meanwhile these enzymes were significantly decreased in the CuF and CuL groups compared to the Cu-treated group, while the positive controls have maintained the same activity of ASAT and ALAT as that of the control (Figure 19).

The ALP activity in the Cu group showed a significant increase when compared to the control, but it decreased significantly in the CuF and CuL rats compared to the Cu group, and demonstrated a non-significant variation in the F and L extracts compared to the control (Figure 20).

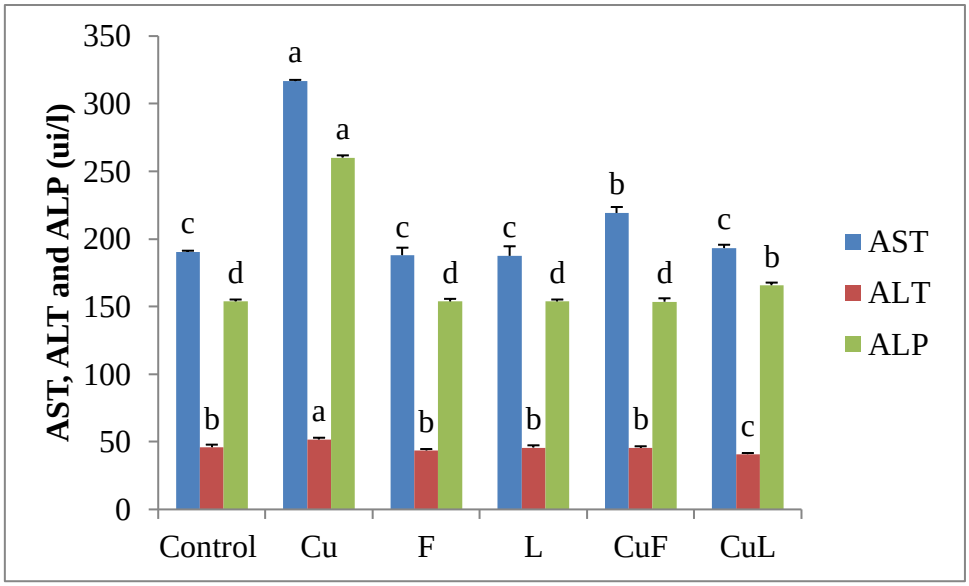

Figure 20: variation of ASAT, ALAT and ALP level in the groups treated by Cu, F, L, CuF and CuL for 30 days. Means that do not share the same letter are significantly different at $p<0.05$.

5- Hepatic MDA:

Hepatic MDA level demonstrated a significant increase in Cu-exposed group compared to the control, but the positive groups of *C. monogyna* F and L extracts have preserved MDA level as that of the control (Figure 21).

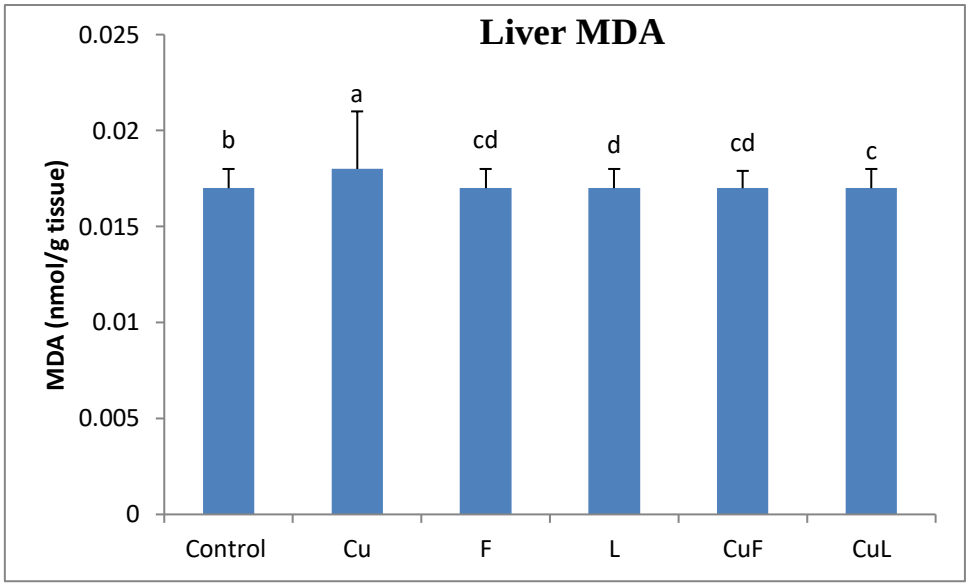

Figure 21: variation of liver MDA level in the groups treated by Cu, F, L, CuF and CuL for 30 days. Means that do not share the same letter are significantly different at $p<0.05$.

6- Hepatic GSH and GPx:

The hepatic GSH concentration and GPx activity were decreased significantly by Cu treatment compared to the control, with a remarkable increase in the combined treatment CuF and CuL compared to the Cu group. However, the positive controls of F and L have showed a significant rise in the levels of GSH and GPx compared to the control (Figure 22 and 23).

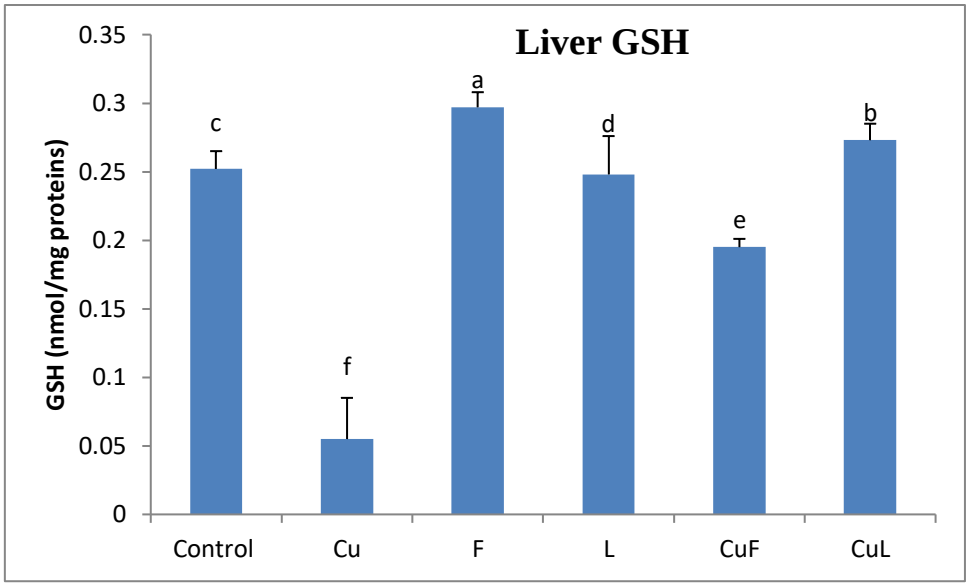

Figure 22: variation of liver GSH level in the groups treated by Cu, F, L, CuF and CuL for 30 days. Means that do not share the same letter are significantly different at $p<0.05$.

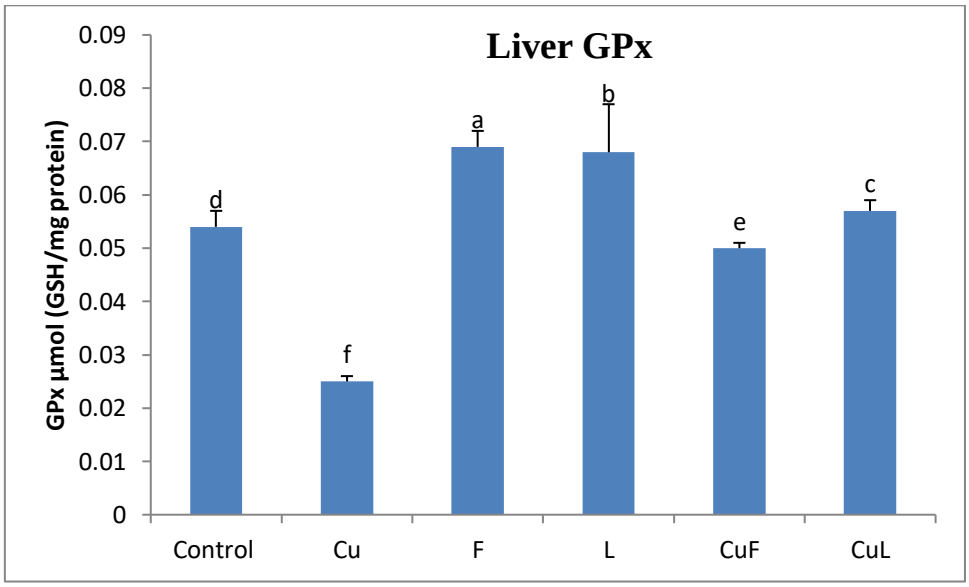

Figure 23. variation of liver GPx level in the groups treated by Cu, F, L, CuF and CuL for 30 days. Means that do not share the same letter are significantly different at $p<0.05$.

7- Renal MDA:

Renal MDA concentration of the Cu group has increased significantly compared to the control, although it has been decreased significantly in rats of the CuF and CuL groups compared to the Cu group. The positive groups F and L were not significantly different than that of the control (Figure 24).

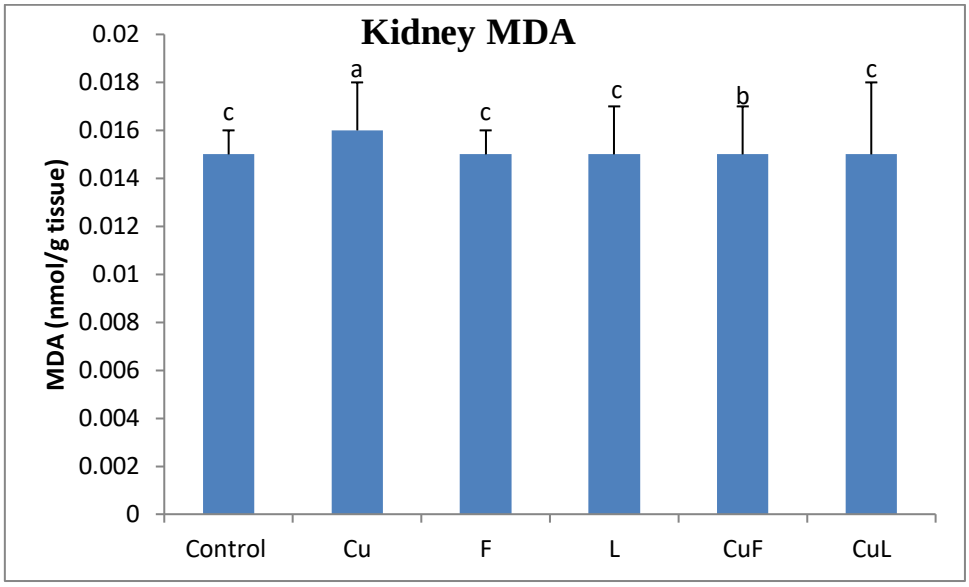

Figure 24: variation of kidney MDA level in the groups treated by Cu, F, L, CuF and CuL for 30 days. Means that do not share the same letter are significantly different at $p<0.05$.

8- Renal GSH and GPx:

Renal GSH level and GPx activity have been decreased significantly in the Cu group compared to the control, but when compared to the Cu group, the two markers of CuF and CuL groups were highly significant. Interestingly, the positive group F of hawthorn extracts has higher GSH and GPx levels than that of the control (Figure 25 and 26).

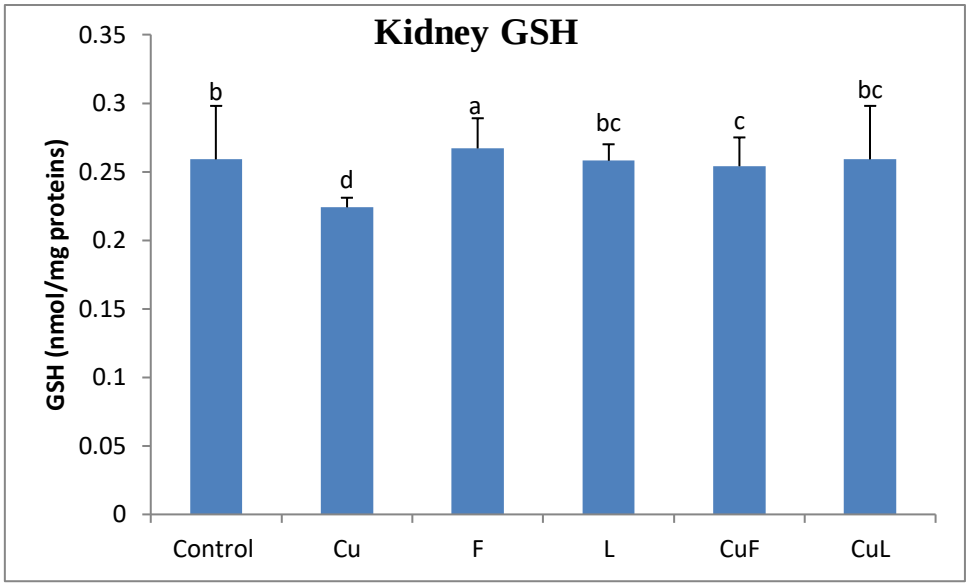

Figure 25: variation of kidney GSH level in the groups treated by Cu, F, L, CuF and CuL for 30 days. Means that do not share the same letter are significantly different at $p<0.05$.

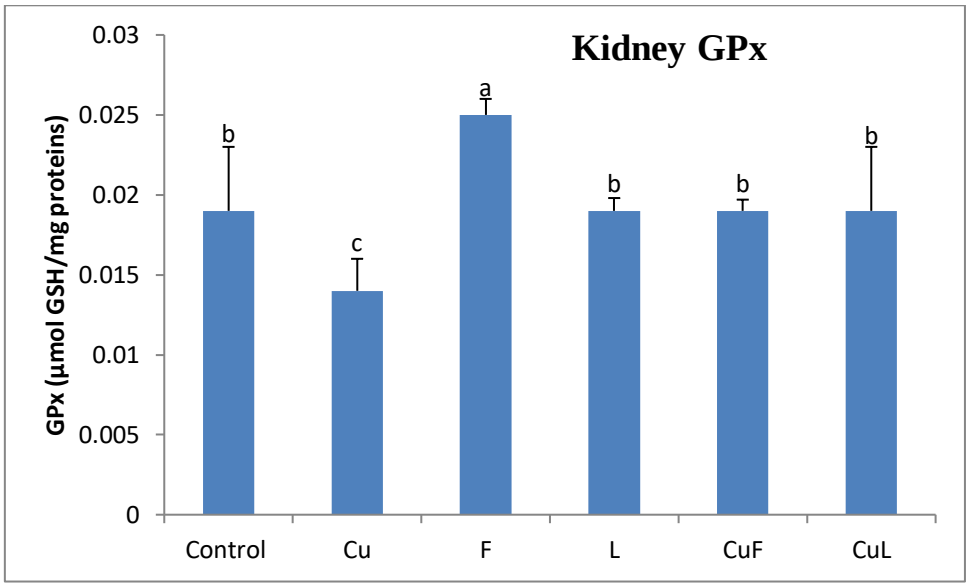

Figure 26: variation of kidney GPx level in the groups treated by Cu, F, L, CuF and CuL for 30 days. Means that do not share the same letter are significantly different at $p<0.05$.

9- Triglycerides:

The data of this experiment led to a pronounced decline in triglycerides in the group treated with Cu compared to the control, but it increased significantly in the CuF and CuL groups compared to the Cu, while triglycerides of the positive groups have decreased significantly compared to the control (Figure 27).

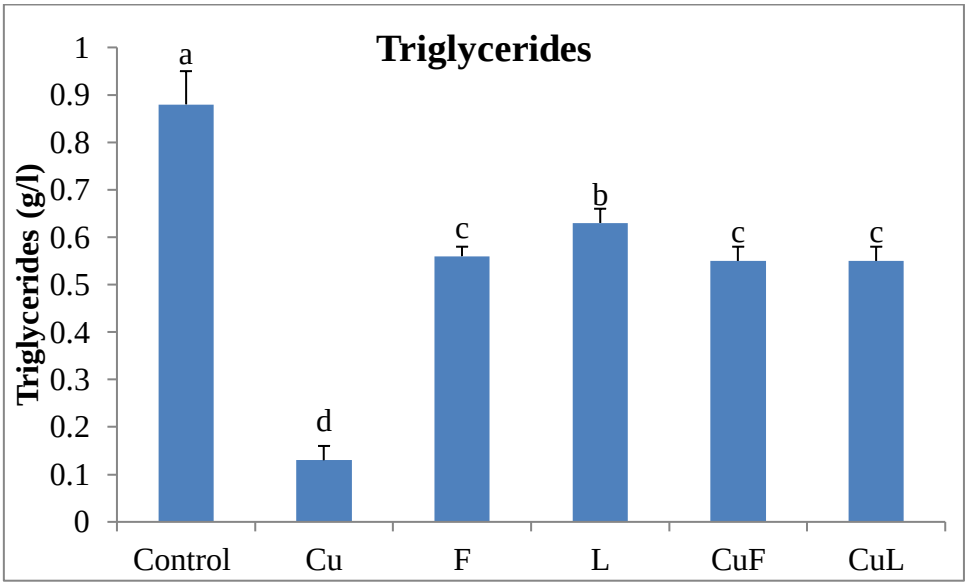

Figure 27: variation of triglycerides level in the groups treated by Cu, F, L, CuF and CuL for 30 days. Means that do not share the same letter are significantly different at $p<0.05$.

10- Cholesterol:

The cholesterol in the group treated with Cu showed a significant decrease compared to the control, but it increased significantly in the CuF and CuL groups compared to the Cu. The positive groups treated with F and L has a significant decrease in cholesterol concentration compared to the control (Figure 28).

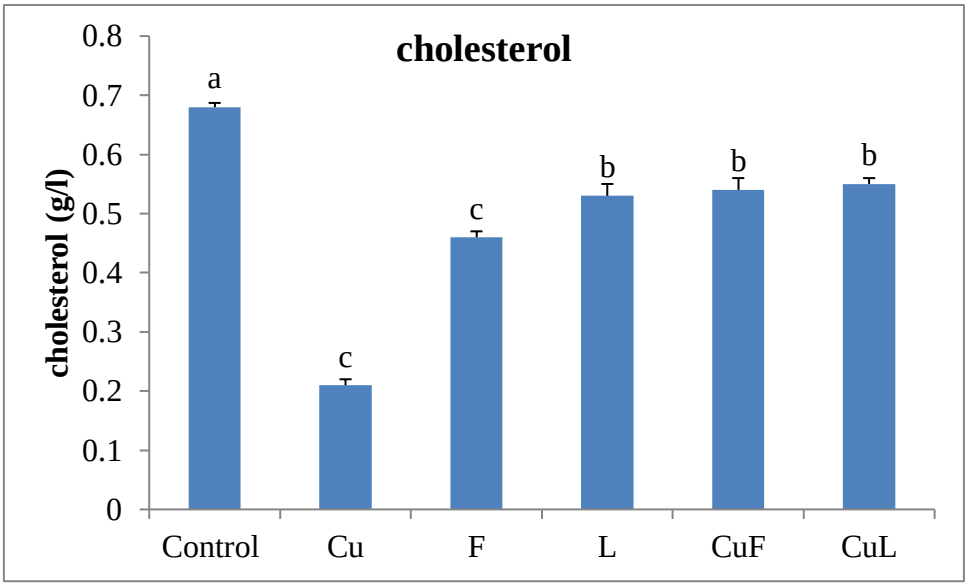

Figure 28: variation of cholesterol level in the groups treated by Cu, F, L, CuF and CuL for 30 days. Means that do not share the same letter are significantly different at $p<0.05$.

11- High density lipoproteins:

High density lipoproteins HDL of the Cu group showed almost the same level as that of the control, but it increased significantly in the CuF and CuL groups compared to the Cu. The HDL level of the positive groups F and L have increased significantly compared to the control (Figure 29).

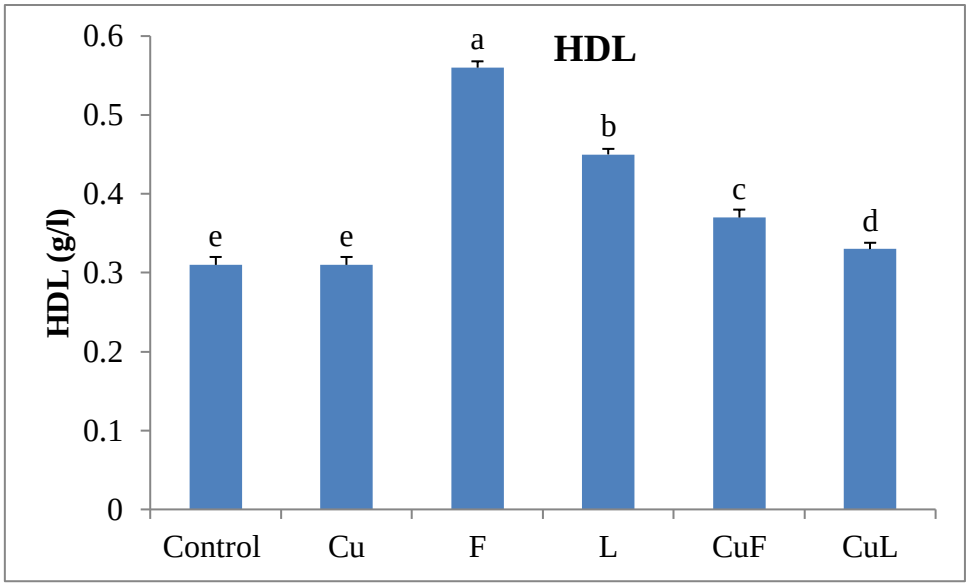

Figure 29: variation of high density lipoprotein level in the groups treated by Cu, F, L, CuF and CuL for 30 days. Means that do not share the same letter are significantly different at $p<0.05$.

12- Low density lipoproteins:

Low density lipoproteins LDL in the group treated with Cu showed a slight but not considerable decrease compared to the control, but it decreased significantly in the CuF and CuL groups compared to the Cu, while it decreased significantly in the two positive controls compared to the control (Figure 30).

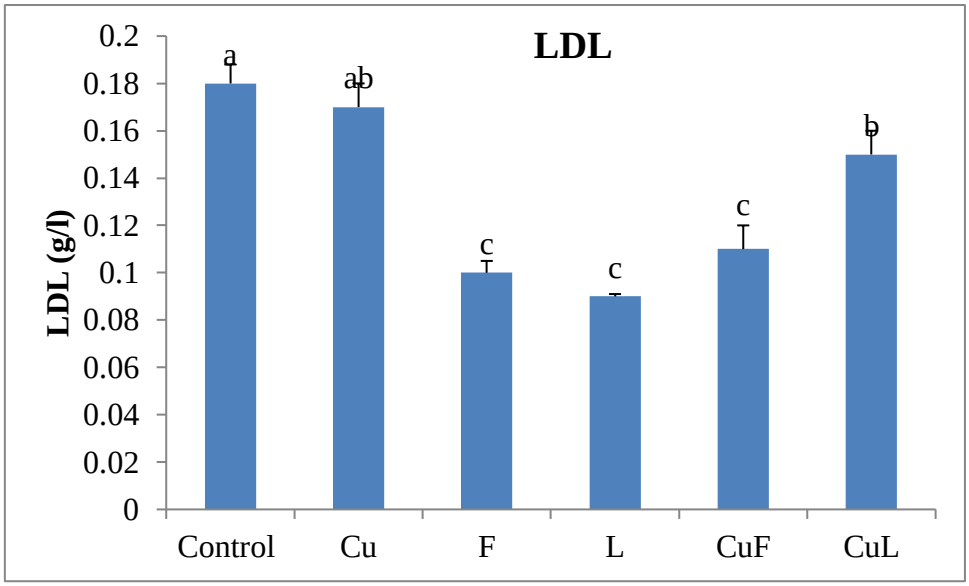

Figure 30: variation of low density lipoprotein level in the groups treated by Cu, F, L, CuF and CuL for 30 days. Means that do not share the same letter are significantly different at $p<0.05$.

DISCUSSION

Total body weight in this finding decreased in wistar rats exposed to copper sulphate after a period of thirty consecutive days. Earlier, Haywood, (1979) demonstrated that copper may reduce the total body weight of rat, as well as Akomolafe et al., (2014) who found a decrease in body weights after exposing rat to 100mg/kg/day for 14 days of copper sulphate. Contrary, others workers reported increased body weight and adipose tissues of Wistar rats after 90days of administration of 9.76 mg/l CuSO4 in drinking water (Tinkov et al., 2012).

In this study plasma urea and creatinine of rats increased significantly in the Cu group, which is in-line with the study Sinkovic et al., (2008) that showed that renal dysfunction is related to copper exposure. Copper is known to induce proximal tubular necrosis while hemoglobin maybe observed in the tubules (Aggrawal, 20016; Biswas, 2019). Furthermore, the administration of 3g of Cu/kg for 5 weeks may induce tubular necrosis (Haywood et al., 1985). While it is confirmed that acute kidney injury by copper and the augmentation of plasma urea level are caused by the tubular cells death (Bauer, 1975). Along with other studies that showed that $CuSO_4$ increased the concentration of plasma urea and creatinine, which might be explained by the inability of kidneys to rid of waste products (Emad and Shimaa, 2016). Likewise, after 5 days of treatment with 200mg/kg of nanocopper, death of renal proximal tubule cells was noticed along with a rise urea and creatinine in blood (Liao and Liu 2012). The lipid peroxidation induced by copper (Indquist, 1968) may lead to kidney injury by provoking necrosis for different cells, as well as free radicals produced by copper may lead to renal oxidative stress (Manzl et al., 2004).

On the contrary, blood urea and creatinine have not been affected after treating rats either with 100-200mg/kg bw of copper sulphate (Akomolafe et al., 2014) or with 50 µmol/kg bw of

inorganic copper for 30 days (Abou-seif et al., 2003), but it was proven that a few cases of acute renal failure were observed in human after an exposition to copper (Kiss et al., 1998).

In our study, copper group has increased the activity of plasma AST ALT and ALP after four weeks exposure as what has been revealed by Akomolafe et al., (2016) and Emad and Shimaa, (2016), while the activities of these enzymes were not affected after administrating rats with 750 µg/g copper sulfate (Aburto et al., 1999). Thus, high level of copper ions may cause hepatic cells' necrosis, leading to leaking of these enzymes in the blood (Naik and Panda, 2008; Rajesh and Latha, 2004). A positive correlation between hepatic copper concentration and liver dysfunction after the treatment of rats with 100mg/kg bw and 200mg/kg of copper sulphate (Kumar et al., 2016). *In vivo,* free copper ions may combine to sulphydryl groups, nucleic acids, and tubulin affecting however, cellular functions mainly enzyme activities, protein synthesis, and intracellular transport (Nederbragt et al., 1984), In addition, a high level of free copper ions may lead to cellular dysfunction by causing lipid peroxidation (Indquist, 1968) leading to lysosomal membrane damage and the liberation of proteolytic enzymes responsible for protein breakdown (Nederbragt et al., 1984).

Copper overload was found to produce ROS (Roy et al., 2009), via the redox cycling Haber-Weiss reaction (Manzl et al., 2004), which may inhibit the entrance of calcium ions and provoking oxidative stress that lead to cells damages (Viarengo and Nicotera, 1991); this may explain the augmentation of MDA level in both liver and kidney of copper-group in the current study. In the study of Ozcelik et al., (2003), the MDA concentration of liver tissues of Sprague–Dawley have increased significantly after 4 weeks with a dose of 100 µg/Ml of copper, also GSH level decreased significantly after the copper treatment (Ozcelik et al., 2003).

Furthermore our results showed a significant decrease in GSH level and GPx activity; admittedly these two markers play a protective role against metal toxicity. It seems that GSH is likely to be consumed by while scavenging free radicals induced by copper ions, leading to a depletion of GSH and GPx as markers of cell defense (Roy et al., 2009).

Our study showed that after a treatment with a toxic dose of copper for a period of 4 weeks, a significant decrease in the levels of triglycerides and cholesterol was observed, while HDL and LDL have not been affected. Copper overload could reduce the concentration of triglycerides (Al Ankari et al., 1998; Babaknejad et al., 2015) and total cholesterol (Mondal et al., 2007). Maintaining the level of LDL and HDL in the copper group perhaps is related to the synthesis of HDL from LDL by modulating HMG-CoA reductase activity (Mondal et al., 2007). Contrary, copper was found to increase the level of cholesterol in cows supplemented with 40mg/kg (Engle et al., 2001). In addition, increased cholesterol and LDL levels were observed as a result of oxidative stress (Galhardi et al., 2004).

In this finding, both fruits and leaves have increased the antioxidant system in liver and kidneys. *C. monogyna* is rich in nutrients and antioxidants which could protect hepatic and nephrotic cells from the damage provoked by copper. Recently, many studies demonstrated that hawthorn has a powerful antioxidant (Shortle et al., 2014) and free radicals scavenger (Bernatonienė et al., 2008), admittedly phenols, oils and vitamins (Bechkri et al., 2017) that may enhance the antioxidants enzymes (Wang et al., 2011). Others confirmed that *C. monogyna* could scavenge the superoxide anions, hydroxyl radicals, hydrogen peroxides and reduce lipid peroxidation (Rice-Evans, 2004). Vitamin C as an electron donor and reducing agent is a potential antioxidant (Sebastian et al., 2003).This vitamin in *C. monoyna* protects against copper toxicity and acts as an antioxidant to prevent lipid peroxidation and protein oxidation in human plasma in vitro (Suh et al., 2003). Moreover, polyphenols were demonstrated to strengthen the antioxidant system (Saibabu et al., 2015), while it induce the

ARE-dependent antioxidant/detoxifying phase II enzymes such as glutathione reductase and glutathione peroxidase by activating the transcription factor Nrf2 through JNK- mediated phosphorylation (Var`I et al., 2011), in addition to reducing the MDA level to protect cells from lipid peroxidation (Saibabu et al., 2015).

The antioxidant effect of *C. monogyna* against copper free radicals and as antioxidant enhancer (Kirakosyan et al., 2003) may explain its role in maintaining the levels of AST, ALT, PAL, urea and creatinine. The *Crataegus pinnatifida* has been confirmed to have a hepatoprotective effect through its powerful anti-inflammatory activity (Kao et al., 2005), while vitamins, zinc and iron of *C. monogyna* could reduce the absorbance of copper in the stomach, giving more protection against copper overload (Ozcan et al., 2005).

C. monogyna has reduced the levels of triglycerides, cholesterol, and LDL, while it enhanced the production of HDL. In fact after the treatment with 2%, hawthorn has significant hypocholesterolemic and vasoprotective activities in rats after hypercholesterolemic diet (Kwok et al., 2010). Researchers found that the alcoholic extract of the *C. monogyna* berries lowered significantly cholesterol, triglycerides and LDL levels (Kausar et al., 2012). *C. monogyna* increased the capacity of the receptors to bind to LDL and preventing the augmentation of cholesterol (Kausar et al., 2011) and so enhancing cholesterol degradation to bile (Rajendran et al., 1996). Hawthorn has been found to decrease the serum levels of cholesterol, LDL-cholesterol, and triglycerides in hypercholesterolemic and atherosclerotic animals (Chang et al., 2002). Also studies showed that hawthorn may lower the body weight as our results showed and used to treat obesity and weight control (Kausar et al., 2012).

In the combined treatments of CuL and CuF groups, the level of HDL increased significantly, which means that hawthorn has a beneficial effect explained by the presence of

catalytic metal ions that increase the long and short chain cholesterol ester and phospholipids (Abuja and Albertini 2001), while the LDL level decreased significantly, since it can be more oxidized by HDL with high copper concentration (Raveh et al., 2001).

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CHAPTER 5

HEMATOLOGY

RESULTS

1- Erythrocytes G6PD:

Results of glucose-6-phosphate dehydrogenase activity indicated that the treatment of rats with Cu has caused a significant decrease comparing to the control, but it increased significantly in the CuL and CuF groups compared to the Cu group, while the positive groups treated with *C. monogyna* extracts fruits F has augmented significantly, but that of leaves L extracts has preserved the same level of G6PD as that of the control (Figure 31).

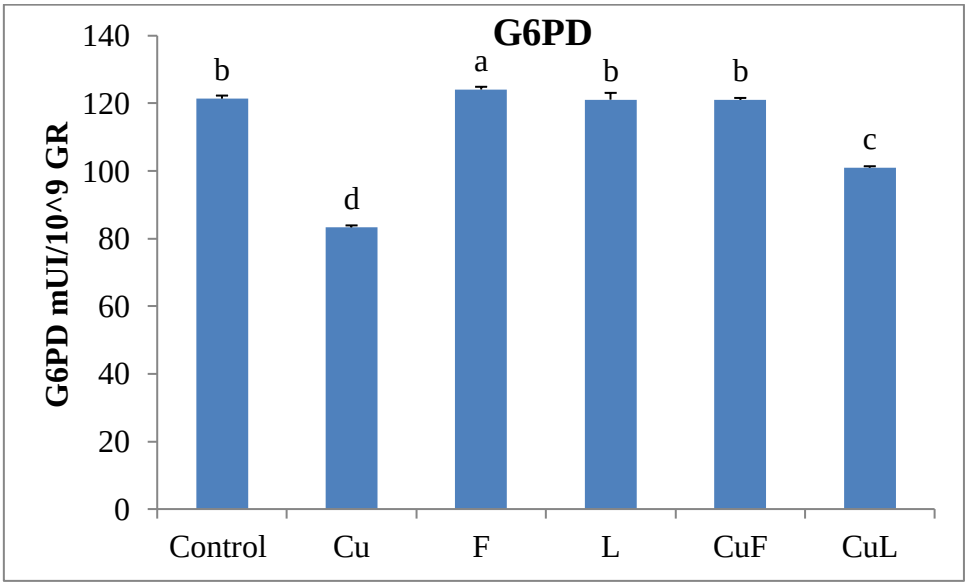

Figure 31: the activity of glucose-6-phosphate dehydrogenase in the rat groups treated by Cu, F, L, CuF and CuL Cu, F, L, CuF and CuL for 30days. Means that do not share the same letter are significantly different at $p<0.05$.

2- White blood cells:

Results of white blood cells' count showed a significant increase in the group treated with Cu compared to the control, but it decreased significantly in the CuL and CuF groups compared to Cu group, while the positive groups treated with fruits F and leaves L extracts have conserved the same level of WBC comparing to the control (Figure 32).

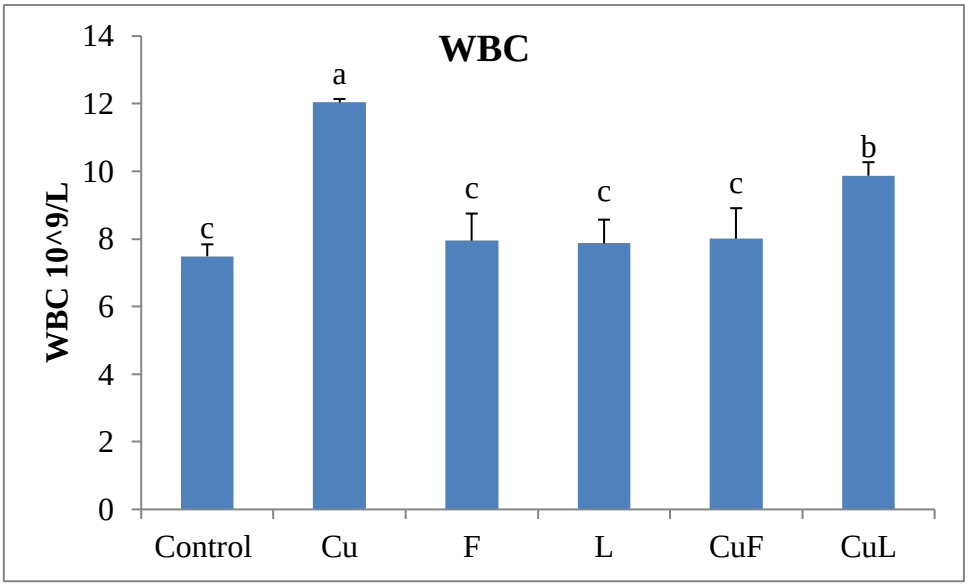

Figure 32: the count of white blood cells in the rat groups treated by Cu, F, L, CuF and CuL Cu, F, L, CuF and CuL for 30days. Means that do not share the same letter are significantly different at $p<0.05$.

3- Red blood cells:

Red blood cells' counts demonstrated a significant decrease in the group exposed to Cu compared to the control, but it increased significantly in the combined administration of F and L. when compared to the Cu-induced group (Figure 33).

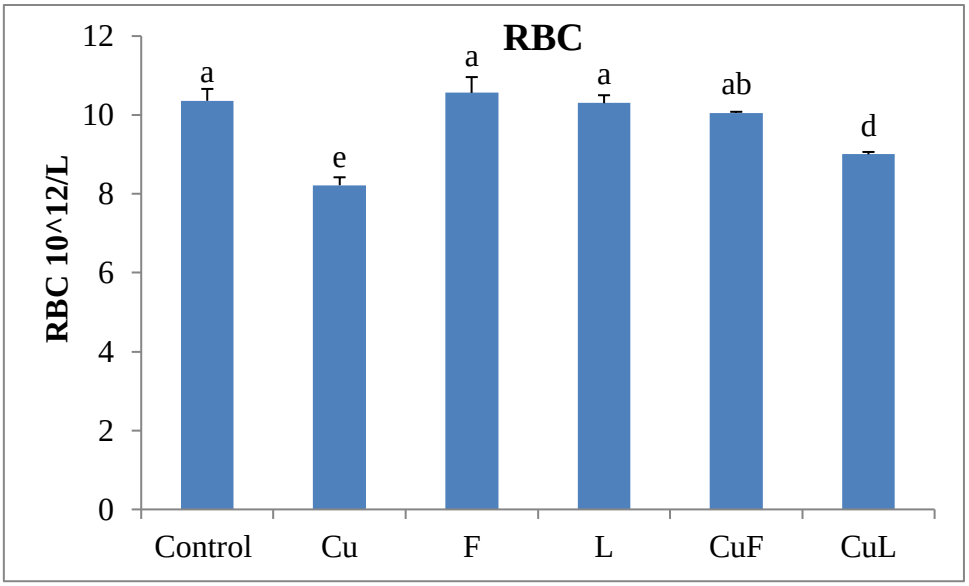

Figure 33: the count of red blood cells in the rat groups treated by Cu, F, L, CuF and CuL Cu, F, L, CuF and CuL for 30days. Means that do not share the same letter are significantly different at $p<0.05$.

4- Platelets:

Platelets' counts showed a significant increase in the group treated with Cu comparing to the control, but it decreased significantly in the CuL and CuF groups compared to the Cu group (Figure 34).

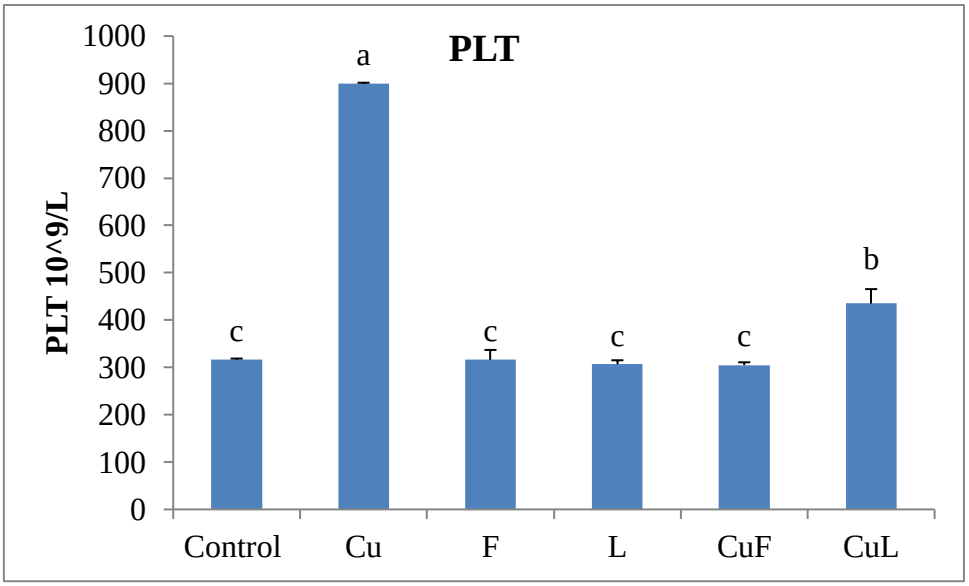

Figure 34: the count of platelet in the rat groups treated by Cu, F, L, CuF and CuL Cu, F, L, CuF and CuL for 30days. Means that do not share the same letter are significantly different at $p<0.05$.

5- Hemoglobin:

Hemoglobin concentration has decreased significantly in the Cu group when compared to the control. Hemoglobin level was significantly higher in rats of CuL and CuF groups compared to the Cu group (Figure 35).

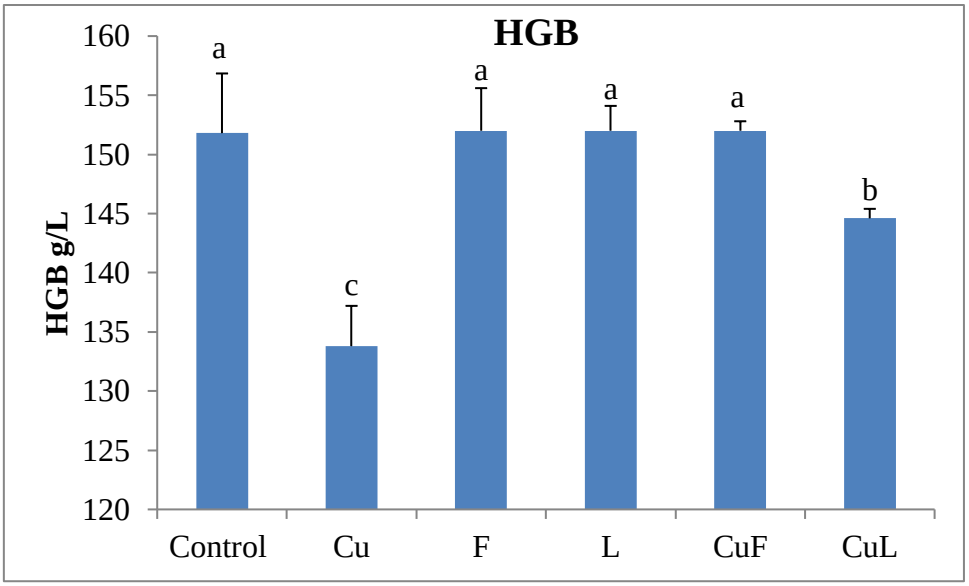

Figure 35: the level of hemoglobin in the rat groups treated by Cu, F, L, CuF and CuL Cu, F, L, CuF and CuL for 30days. Means that do not share the same letter are significantly different at $p<0.05$.

6- Hematocrit:

Hematocrit level of the Cu group has declined significantly compared to the control, but it raised significantly in the CuL and CuF groups compared to the Cu group, while the positive groups treated with *C. monogyna* extracts fruits F has increased, but that of leaves L has maintained the same level of HCT as that of the control (Figure 36).

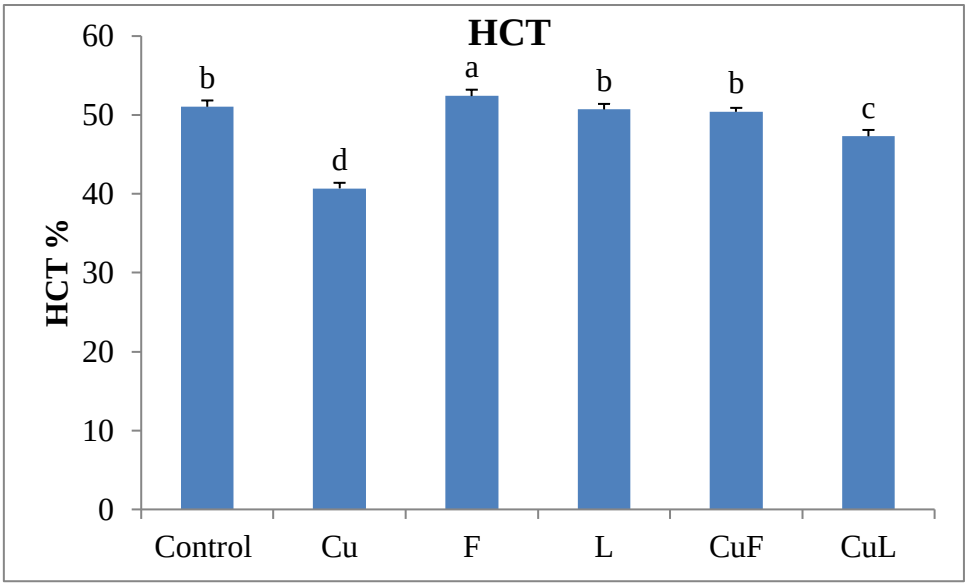

Figure 36: the level of hematocrits in the rat groups treated by Cu, F, L, CuF and CuL Cu, F, L, CuF and CuL for 30days. Means that do not share the same letter are significantly different at $p<0.05$.

7- Mean corpuscular volume:

Mean corpuscular volume level of the Cu-treated rats demonstrated a significant decrease as compared to the control, whereas it showed a significant increase in the CuL and CuF extracts compared to the Cu group (Figure 37).

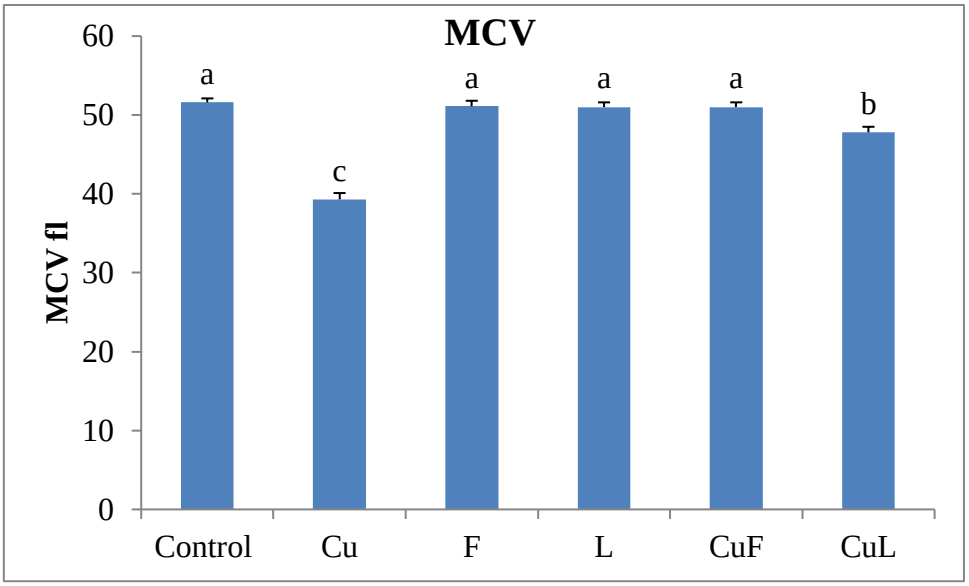

Figure 37: the level of mean corpuscular volume in the rat groups treated by Cu, F, L, CuF and CuL Cu, F, L, CuF and CuL for 30days. Means that do not share the same letter are significantly different at $p<0.05$.

DISCUSSION

Copper in this finding has decreased the levels of G6PD, RBC, HGB and HCT, while it increased WBC, PLT and MCV. Other data reported that copper overload could decrease RBC and HGB (Akomolafe et al., 2014), which resulted from red blood hemolysis induced by the free copper ions. The lower activity of G6PD may be explained the inhibiting affect of copper on the enzyme (Joshi et al., 2002) responsible on the protection of red cells from the oxidative stress by maintaining physiological GSH level, therefore the G6PD deficiency can make red blood cells prone to oxidative stress. Other authors postulated that toxic copper may cause hemolysis and red blood dysfunction in rats (Savaru et al., 2007) because of erythropoiesis alterations through antagonist effect where high load of copper could reduce iron absorption in the intestinal tract (pmila et al., 1991) and also can provoke anemia (Eck et al., 1989) or iron storage and methaemoglobinaemia (Oldenquist and Salem1999). Hence, as copper is involved in erythropoiesis (Samanta et al., 2011), high level of the metal possibly causes a negative feedback and reduce the blood cells formation. Moreover, the MCV and HCT have been increased significantly by copper in rats (Akomolafe et al., 2016), without affecting RBC and HBG levels (Akomolafe et al., 2016). Liver injury by the copper may lead to coagulation cascade (Nelson, 2002); this my explain the observed raise in the PLT levels of the current investigation, but contrary to our results, high level of copper in rats has decreased the PLT counts through inhibiting the production of thrombopoietin (Ganong, 2009).

Copper may lead to inflammatory reactions to some organs such as liver, heart and kidneys, which may explain the augmentation of WBC counts that are known to play major role in immunity reactions such as macrophages, which are sensitive to heavy metals' toxicity (Witeska and Wakulska, 2007).

The co-administration of hawthorn fruits and leaves extracts have particularly increased the blood G6PD, RBC, HMG, HTC and MCV levels, but it reduced WBC and

platelets of rats after one month trial. In this study, *C. monogyna* extracts perhaps play an important role in scavenging free radicals induced by copper ions, as the other studies that demonstrated that aqueous and ethanolic extracts had the capacity in protecting cells from oxidative stress (Bernatonienė et al., 2008) by strengthening the antioxidant system trough the enhancement of G6PD synthesis to protect blood cells. In one hand, many results confirmed that hawthorn is rich in polyphenols (Liu et al., 2019), in the other hand, polyphenols and flavonoids play a protective role for hematological markers against lead toxicity (Aksu et al., 2012). Too, the anti-inflammatory activity of *C. monogyna* may be the reason of maintaining WBC count close to their normal level in our findings (Kumar et al., 2012)

Omega-3 present in *C. monogyna* (Bechkri et al., 2017) may play an important role in keeping physiological levels of blood markers in this study. Thus, omega-3 was reported to protect sickle cells anemia of patients received daily doses for one year (Daak et al., 2013). Further studies confirmed the presence of vitamins in the *C. monogyna* (Özcan et al., 2005) as it was proven to keep healthy hematological markers of certain patients (fishman et al., 2000).

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CONCLUSION

The objective of this research is to explore the protective role of *Crataegus monogyna* in mitigating some physiological disorders induced by the toxic dose of copper administrated orally for 30 days

After the treatment with copper sulfate for 30 days, alterations in reproductive, hepatic, and renal functions, in addition to the lipid profile and hematological markers were observed.

On the other hand, *C .monogyna* aqueous extract of both leaves and fruits was able to reduce the toxic effect of copper, by improving sperm quality and strengthening the organs' antioxidant system.

As perspective, it is interesting to focus on the following points:

- Realizing other extractions of hawthorn and figuring their active compounds.
- Exploring the beneficial effect of hawthorn on male reproduction.
- Looking for the mode of action of copper on synapses.
- Dosage of copper in semen and in the epididymis.

RESEARCH ACTIVITIES

J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 44(3): 452-469, 2020
Doi: 10.33483/jfpau.716103

ORIGINAL ARTICLE / ÖZGÜN MAKALE

PROTECTIVE ROLE OF *CRATAEGUS MONOGYNA* ON SPERM QUALITY AND TESTIS OXIDATIVE STRESS AGAINST COPPER-INDUCED TOXICITY

CRATAEGUS MONOGYNA'NIN, BAKIR KAYNAKLI TOKSİSİTEYE KARŞI SPERM KALİTESİ VE TESTİS OKSİDATİF STRESİ ÜZERİNDEKİ KORUYUCU ROLÜ

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ABSTRACT

Objective: *The purpose of this study was to investigate the possible beneficial use of common hawthorn Crataegus monogyna aqueous extract at 1.5g/Kg bw/day against chronic copper sulfate intoxication (100mg/Kg bw) in Wistar rats.*

Material and Method: *Animals were divided into 6 groups; the untreated control (C), 2 positive controls treated respectively with hawthorn fruits (F) and leaves (L), 1 group treated with copper (Cu) and 2 combined treatment groups treated with Cu and hawthorn-fruits (CuF) and Cu and hawthorn-leaves (CuL). After 30 days of oral administration, testis weight and plasma testosterone levels were evaluated, in addition to the epididymal sperm concentration, motility, vitality, velocity (VCL, VSL and VAP), the amplitude of lateral head displacement (ALH), and the beat cross frequency (BCF). Testicular glutathione (GSH), malondialdehyde (MDA), and glutathione peroxidase (GPx) were also evaluated.*

Result and Discussion: *Cu exposure reduced testosterone, sperm concentration, live sperm, VCL, VSL, VAP, ALH, BCF, GSH, and GPx levels compared to control groups. Dead sperm and MDA levels were increased in rats of Cu group compared to the untreated control. When compared to the Cu group, levels of testosterone, sperm concentration, sperm motility, live sperm, VCL, VSL, VAP, ALH, BCF, GSH, and GPx were much higher in the CuF and CuL groups, along with a significantly lower MDA concentration. In conclusion, hawthorn, when co-administered as an aqueous extract with Cu, protected most biological markers against copper toxicit, while positive control (s) boosted sperm concentration and velocity (VCL and VAP).*

Keywords: *C. monogyna, CASA, Copper, sperm, testosterone*

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Submitted / Gönderilme: 07.04.2020 **Accepted / Kabul:** 30.08.2020

ÖZ

Amaç: *Bu çalışmanın amacı, Wistar sıçanlarında, kronik bakır sülfat zehirlenmesine (100mg / Kg bw) karşı yaygın alıç Crataegus monogyna sulu ekstraktının (1.5g / Kg bw/ gün) olası faydalı kullanımını araştırmaktır.*

Gereç ve Yöntem: *Hayvanlar, tedavi edilmemiş kontrol (C), sırasıyla alıç meyveleri (F) ve yaprakları (L) ile muamele edilmiş 2 pozitif kontrol, bakır (Cu) ile muamele edilmiş grup ve Cu ve alıç meyveleri (CuF) ve Cu ve alıç yaprakları (CuL) ile muamele edilmiş 2 kombine tedavi grubu olmak üzere 6 gruba ayrılmıştır. 30 günlük oral uygulamadan sonra, epididimal sperm konsantrasyonu, motilite, canlılık, hız (VCL, VSL ve VAP), dış yana baş deplasmanının amplitüdü (ALH) ve sperm kuyruğu vuruş sıklığının (BCF) yanısıra testis ağırlığı ve plazma testosteron seviyeleri değerlendirilmiştir. Ayrıca, testis glutatyonu (GSH), malondialdehid (MDA) ve glutatyonperoksidaz (GPx) da değerlendirilmiştir.*

Sonuç ve Tartışma: *Cu maruziyeti, kontrol gruplarına kıyasla testosteron, sperm konsantrasyonu, canlı sperm, VCL, VSL, VAP, ALH, BCF, GSH ve GPx seviyelerini azaltmıştır. Cu grubunun sıçanlarında ölü sperm ve MDA seviyeleri, tedavi edilmeyen kontrole kıyasla artmıştır. Cu grubuyla karşılaştırıldığında, testosteron seviyeleri, sperm konsantrasyonu, sperm motilitesi, canlı sperm, VCL, VSL, VAP, ALH, BCF, GSH ve GPx seviyeleri CuF ve CuL gruplarında çok daha yüksek ve MDA konsantrasyonları ise anlamlı olarak düşüktür. Sonuç olarak, alıç, Cu ile sulu bir özüt olarak uygulandığında, bakır toksisitesine karşı çoğu biyolojik belirteci korumuş ve sperm konsantrasyonunu ve hızını (VCL ve VAP) artırmıştır.*

Anahtar Kelimeler: *Bakır, C. monogyna, CASA, sperm, testosteron*

INTRODUCTION

Copper is a trace element and important component of numerous metalloenzymes that are involved in energy and antioxidant metabolisms. However, some of copper's chemical forms, such as copper sulfate are very toxic [1]. Copper binds to binding proteins in the bloodstream and, distributes to all tissues especially the brain and the liver, which can secrete the excess of the metal into bile. Hypercupremia may cause several oxidation reactions, inflammations, and tissues damage by inducing free radical generations [2]. Copper can enter the body orally through food, by inhalation into the lungs or through the skin by direct contact. Copper is used in agriculture as a fungicide, herbicide, and insecticide [3]. Also, it is used as an electrical conductor in several industries, it has many chemical applications, and is known as a coinage metal. In nature, copper exposure may be caused by clouds of dust, volcanoes and forest fires. Copper dyshomeostasis has been linked to a variety of disorders. For example, ATP7A and ATP7B are both involved in copper metabolism; mutations of the former lead to Menkes' disease, but that of the latter causes Wilson's disease [4].

Previous studies showed that human spermatogenesis has decreased by up to 60% after 40 years [5]. In addition, many couples today are infertile [6]; this male sexual disorder may be related to pollutants [7]. The discharge of heavy metals into the environment has led to a harmful deterioration of the ecosystems [8]. Copper can cause several problems both in excess and deficiency. Copper imbalance in both men and women might affect reproduction, and women are said to be copper-dominant and men are zinc-dominant [1]. Excess copper harms male reproduction; metal

contamination provokes certain pathophysiological alterations in humans and animals, affecting sperm quality and causing infertility [9]. In its ionic form, copper is toxic to a variety of cells, including human spermatozoa [10]. According to Wong et al. [11], a positive correlation between blood Cu concentration and sperm motility dysfunction was found. However, cytosolic Cu is mainly bound to metallothioneins that may reduce its toxicity to some extent. The hydroxyl free radicals induced by the Fenton reaction of copper are very destructive to tissues [12] of the testis and epididymis, and also reduce antioxidant biomarkers such as catalase, superoxide dismutase, glutathione, and glutathione peroxidase. This oxidation can change sperm quality by modifying spermatozoa shape and movement. In addition, copper's effect on the pituitary receptor can provoke hormonal imbalance. Copper exposure causes several symptoms such as erectile dysfunction, anxiety, and testicular pain [1]. Furthermore, high copper levels can lead to a decrease in sperm concentration, motility, and vitality [13].

Today, numerous plants are used as remedies in the treatment of many ailments [14]. Hawthorn *Crataegus monogyna* is a very common shrub plant in the Mediterranean basin that is used by the local population in North Africa in certain traditional therapeutic applications including for hypertension, heart disorders, diabetes, anxiety, cancer, and some abdominal symptoms. The Crataegus spp have been used in medicinal treatments and as food [15]. Many studies conducted in Europe, Asia, and the USA showed that hawthorn has a high content of phenolic compounds that show antioxidant activity by scavenging superoxide anions, hydroxyl radicals, hydrogen peroxides, and by reducing lipid peroxidation [16].

This study aims to investigate the possible protective role of common hawthorn *Crataegus monogyna* aqueous extract against the induced toxicity of copper sulfate in Wistar rats by measuring certain reproductive and oxidative stress markers.

MATERIAL AND METHOD

Plant preparation

Fruits and leaves of the common hawthorn Crataegus monogyna were harvested every 3 days from the region of Annaba in the northeast of Algeria in November. Each of the 2 aqueous extracts of fruits (F) and leaves (L) was prepared daily by crushing 1.5g/kg bw (fruits and leaves) in an appropriate volume of distilled water and letting the mixtures streep overnight (12 hours) at room temperature in order to obtain 10 ml of filtered solutions of each of fruits and leaves in the morning. The aqueous extracts were administrated to rats per os daily for a period of 30 consecutive days.

Preparation of copper solution

Copper sulfate powder (Cu) was freshly dissolved in distilled water; this solution was administered to animals by gavage at a dose of 100mg/kg bw/day for 30 days.

Experimental design

Wistar rats were purchased from the Pasteur Institute (Algiers), each weighing 196±8 g. Thirty-six males were divided into 6 equal groups: the control (C), the copper group (Cu) which received 100 mg/kg bw/day, the fruits group (F) which received an aqueous extract of 1.5 g fruits/kg bw/day, the leaves group (L) which recieved an aqueous extract of 1.5g leaves/kg bw/day, and 2 other groups that were treated with a combination of copper and fruits (CuF) or copper and leaves (CuL). Rats received tap water and standard diet ad libitum. After 30 days of continuous treatment, animals were sacrificed by decapitation; the blood was collected in heparinized tubes and then centrifuged at 3000 rpm for 10min. The plasma obtained was stored at -20 °C along with the testes, which had already been weighed, till further analysis. Animals' treatments were authorized by the Ethical Committee of Animal Sciences at the University of Badji Mokhtar-Annaba, before starting the experimental work.

Measurement of testosterone

The Ultrasensitive TESTOSTERONE ELISA test (DRG instrument GnbH) is based on the principle of a solid phase enzyme-linked immunosorbent assay. The assay system utilizes a unique monoclonal antibody directed against a district antigenic determinant on the intact testosterone molecule. Mouse monoclonal anti-testosterone antibody was used for solid phase (microtiter wells) immobilization, and goat anti-testosterone antibody was used in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample was allowed to react simultaneously with the antibodies. After 2 hours of incubation at room temperature with shaking, the solid phase and enzyme were washed with distilled water to remove unbound labeled antibodies. A solution of tetramethylbenzidine (TMB) was added and incubated for 20 minutes, resulting in the development of a blue color. The color development was stopped with the addition of 1N HCl, and the resulting yellow color was measured using a spectrophotometer at 450 nm. The concentration of testosterone was directly proportional to the color intensity of the sample.

Semen analysis

Semen analysis was realized using the Computer-Assisted Sperm Analysis Method (CASA) using Sperm Class Analysis (SCA®, Microptic, Barcelona, Spain). The epididymal semen was

obtained immediately after sacrifice, and then a drop of semen (about 1µl) was diluted with a physiological solution of NaCl 0.09%, and 5µL of the mixture was placed in an empty chamber slide (GoldCyto model). The slide was then placed on a Nikon Eclipse (Nikon E200-LED) microscope at the phase objective (x4). The sperm markers of concentration, motility, vitality linearity (VCL, VSL), velocity (VAP), the amplitude of lateral head displacement (ALH) and the beat cross frequency (BCF) were automatically calculated.

Hypo-osmotic swelling (HOS) test

The HOST test was used to evaluate the integrity of sperm by exposing a drop of sperm, derived from the epididymis cauda, to the hypo-osmotic solution composed of fructose and sodium citrate [17], after which 100 spermatozoa were observed and the number of live sperms (as shown by an inflation of the tail) were counted.

Measurement of oxidative stress parameters

Frozen stored samples of testis were thawed and 100mg of each sample was taken and transferred to test tubes for the determination of glutathione (GSH) using the method of Cory and Weckbecker [18]. The testicular total proteins were quantified according to the colorimetric method of Bradford [19] by using the Coomassie Brilliant Blue G-250. Malondialdehyde (MDA) was estimated by using the method of Ohkawa et al. [20]. The measurement of glutathione peroxidase (GPx) was realized by the method of Flohe and Günzler [21].

The dosage of glutathione (GSH) was carried out according to the method of Wekbeker and Cory (1988). The principle of this assay is based on the measurement of the optical absorbance of the acid 2-nitro-5-mercapturic. The latter results from the reduction of 5,5'-dithio-bis-2- acid nitrobenzoïque (Ellman's reagent, DTNB) by groups (-SH) of glutathione. For this deproteinization of the homogenate is essential in order to keep only specific thiol groups of glutathione.

The tissues proteins were quantified according to the colorimetric method of Bradford (1967) who uses Gloss Blue Coomassie G250 (BBC) as a reagent and the serum albumin of breef (BSA) as standard. The BBC reacts with the amino groups (-NH2) for protein to form a complex of blue color. The emergence of this color reflects the degree of ionization of the acid and intensity established the concentration of protein which is measured spectrophotometrically at 595nm.

MDA is a product of lipid peroxidation reactions that forms during the attack of polyunsaturated lipids by reactive oxygen species generated by certain contaminants. In our study, testicular MDA levels were assessed using the method of Ohkawa et al (1979). The dosage is based on the formation in an acidic and hot environment (100 ° C) between MDA and thiobarbituric acid

(TBA) of a colored pigment absorbing at 530 nm, extractable by organic solvents like butanol.

The enzymatic activity of glutathione peroxidase (GPx) was measured by the method of Flohe and Gunzler (1984). This method is based on the reduction of hydrogen peroxide (H 2O2) in the presence of reduced glutathione (GSH), the latter is transformed into (GSSG) under the influence of the GPx.

Statistical analysis

Statistics was realized using (MINITAB 18 Software ANOVA Tukey). Results are expressed as mean ±standard deviation. The significant test was considered at $p<0.05$.

RESULT AND DISCUSSION

Testicular weights and testosterone

Results presented in Table 1 showed that differences between the absolute testicular weights of all groups were statistically non-significant when compared to the control. Testosterone concentration (Table 1) was significantly lower in Cu group compared to the untreated control. When compared to the Cu group, testosterone was significantly higher in the CuF and CuL groups. Testosterone levels of the positive control F and L groups were significantly lower compared to the control.

Table 1. Mean testicular absolute weights (g), and testosterone level (ng/ml) in wistar rats treated with copper sulphate and *C. monogyna* leaves and fruits extracts for one month. Results are expressed as mean±SD.

Groups	Testis (g)	Testosterone (ng/ml)
Control	1.67±0.31[a]	5.80±0.007[a]
Cu	1.67±0.015[a]	1.32±0.150[f]
F	1.67±0.01[a]	5.55±0.121[b]
L	1.69±0.02[a]	5.23±0.012[c]
CuF	1.67±0.012[a]	3.10±0.008[e]
CuL	1.67±0.01[a]	4.10±0.003[d]

Means that do not share the same letter are significantly different at $p<0.05$.

Sperm concentration

Results of semen analysis indicated a significant lower sperm concentration in the Cu group compared to the control, Sperm concentration showed no significant difference between the F group and the control but was significantly higher in the L groupthan in the control, and significantlyhigher in the CuF and CuL groups compared to the Cu group (Figure 1).

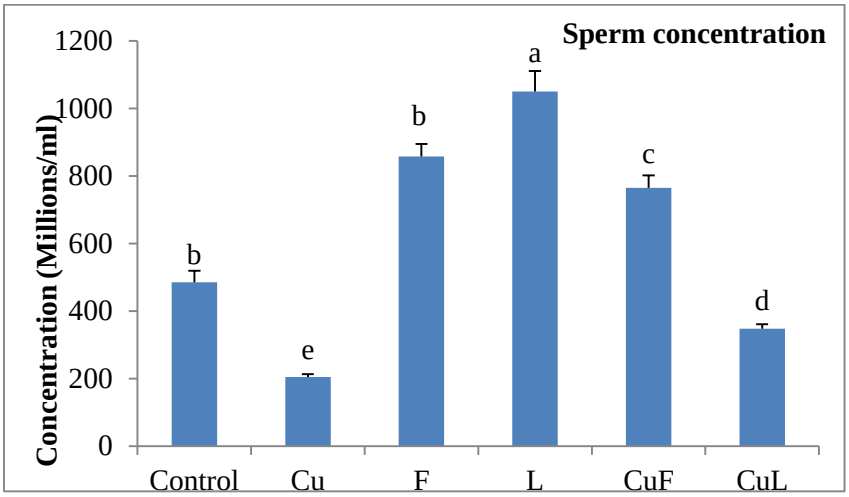

Figure 1. Evaluation of sperm concentration (Millions/ml) of Wistar rats exposed to copper sulphate and *C. monogyna* leaves and fruits for one month. Results are expressed as mean±SD. Means that do not share the same letter are significantly different at $p<0.05$.

Sperm motility

Sperm motility was significantly lower in both the Cu group and the F group compared to the untreated control. Motility was also significantly lower in the CuF and CuL groups compared to the control but higher than the Cu group (Figure 2).

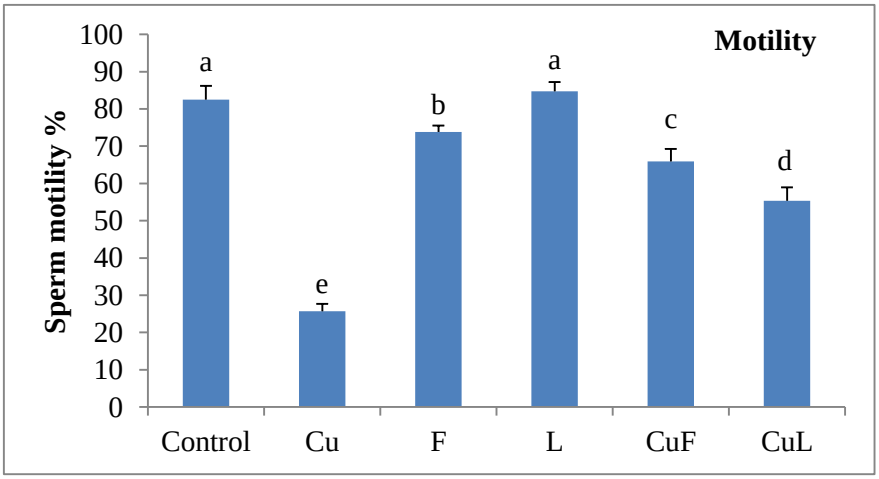

Figure 2. Evaluation ofsperm motility percentage (%) of Wistar rats treated with copper sulphate and *C. monogyna* leaves and fruits for one month. Results are expressed as mean ± SD.Means that do not share the same letter are significantly different at $p<0.05$.

Dead and live sperm

Percentage of dead sperm in the Cu group was significantly higher compared to the control, while F, L, CuF, and CuL group showed levels close to that of the control (Figure 3). On the other hand, percentage of dead sperm was remarkably lower in the combined treatments of CuF and CuL groups compared to the Cu exposed group. Live sperm of the Cu group was slightly decreased when compared to the control, with a weak reduction in the CuF and CuL groups, whereas F, CuF and CuL have kept in close percentage as that of the control (Figure 3).

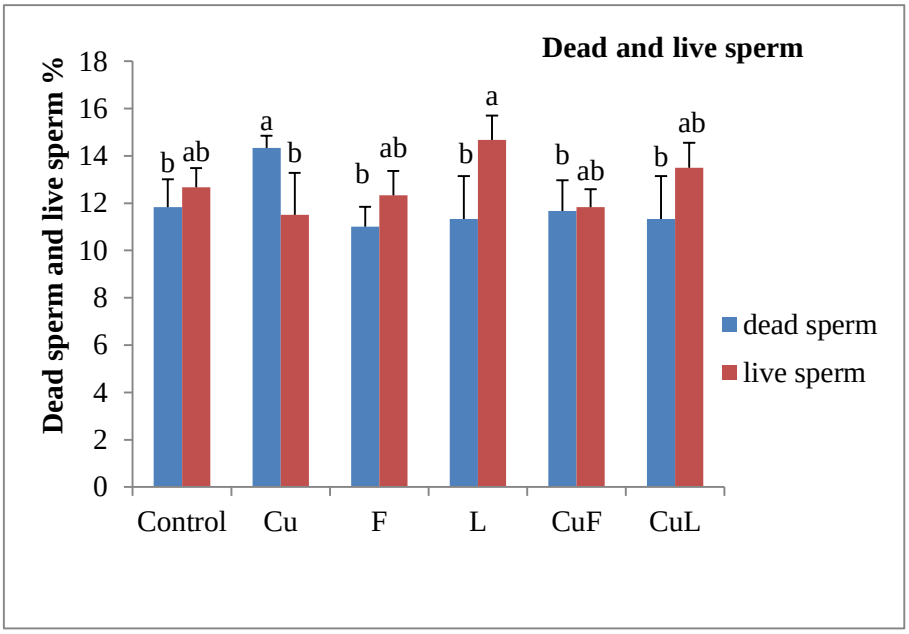

Figure 3. Evaluation of sperm vitality (dead sperm and live sperm percentage %) of Wistar rats exposed to copper sulphate and *C. monogyna* leaves and fruits for one month. Results are expressed as mean ± SD. Means that do not share the same letter are significantly different at $p<0.05$.

Sperm velocity

The VCL, VSL, and VAP of spermfrom the Cu group were significantly lower compared to the control. VCL, VSL, and VAP of spermin the CuF and CuL groups were significantly higher than the Cu group, and not statistically different fromthe control group (Figure 4). The VCL and the VAP of theL positive control were significantly higher than that of the control group.

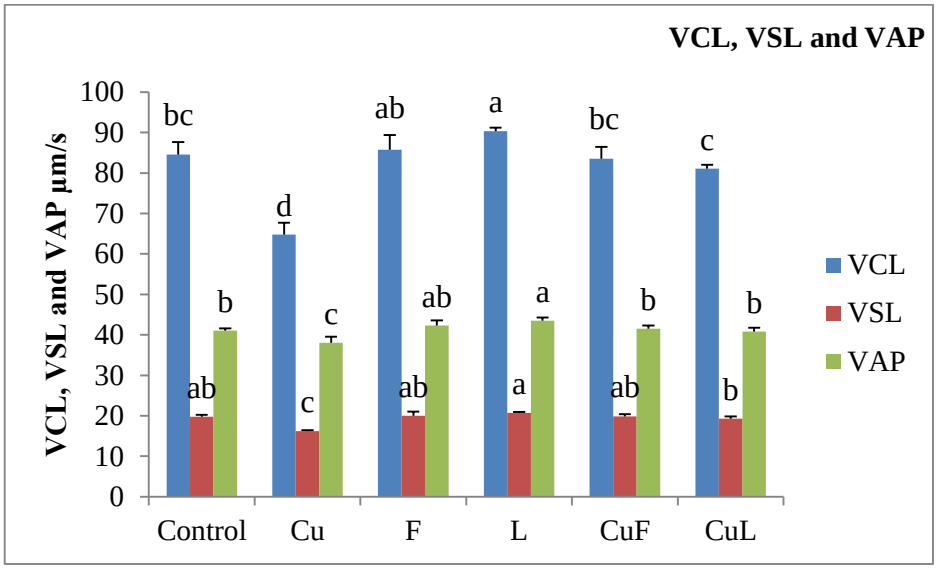

Figure 4. Evaluation of sperm velocity (VCL, VSL and VAP) of Wistar rats exposed to copper sulphate and C. monogyna leaves and fruits for one month. Results are expressed as mean±SD. Means that do not share the same letter are significantly different at $p<0.05$.

Amplitude of lateral head displacement

The sperm ALH was significantly lower in the Cu group compared to the control (Figure 5). Sperm ALH in the other groups were not significantly different from that of the control.

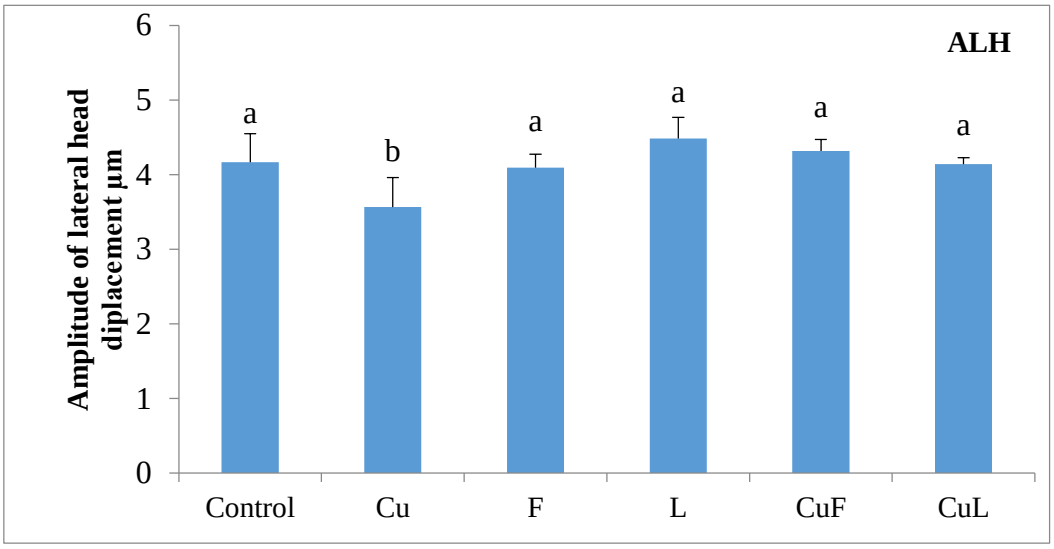

Figure 5. Evaluation of sperm amplitude lateral head displacement (ALH) of Wistar rats exposed to copper sulphate and C. monogyna leaves and fruits for one month. Results are expressed as mean±SD. Means that do not share the same letter are significantly different at $p<0.05$.

Beat cross frequency

The BCF was lower in the Cu group compared to the control, while F, L, CuF and CuL groups did not differ significantly from the control (Figure 6).

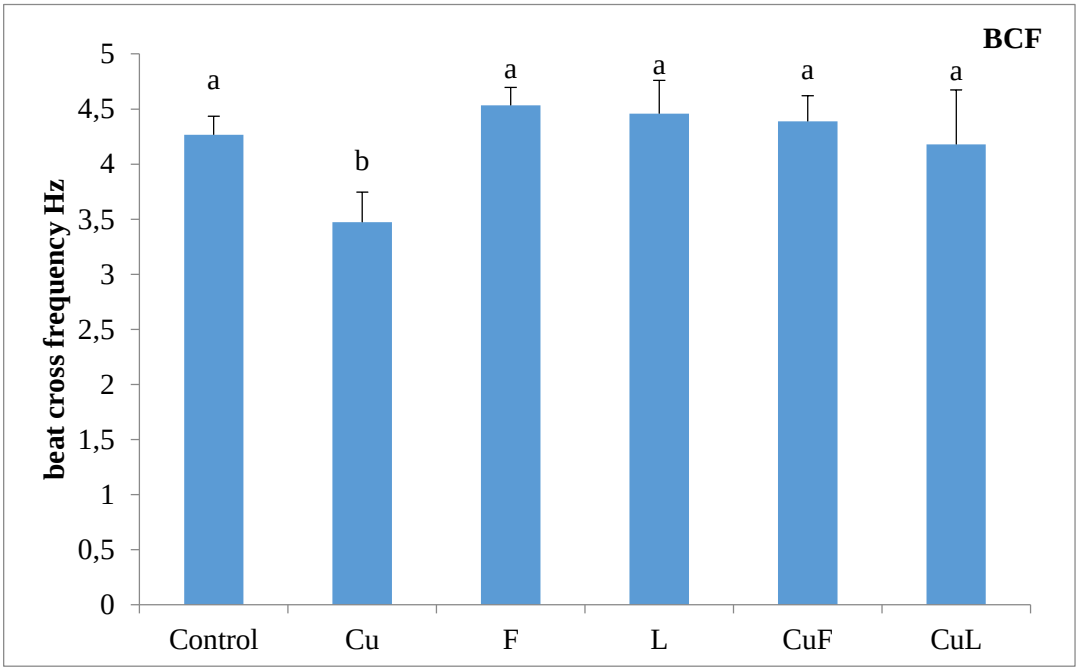

Figure 6. Evaluation of sperm beat cross frequency (BCF) of Wistar rats exposed to copper sulphate and C. monogyna leaves and fruits for one month. Results are expressed as mean±SD. Means that do not share the same letter are significantly different at $p<0.05$.

Oxidative stress markers

The MDA level was significantly higher in Cu group compared to the control group, but it was significantly lower in the CuF and CuL groups compared to the Cu group (Table 2). The CuF group showed significantly higher levels than the control, while the CuL group was not significantly different from the control.

In contrast, the GSH and GPx levels were significantly lower in the Cu group compared to the control. These levels were significantly higher in the CuF and CuL groups compared to both the Cu groupand the control (Table 2). There were no differences in the MDA, GSH and GPx of the positive controls F and L and that of the control.

Table 2. Mean testicular levels of MDA (nmol/g tissue), GSH (nmol/mg proteins), and GPx (µmol GSH/mg proteins) of wistar rats treated with copper sulphate and C. monogyna leaves and fruits for one month. Results are expressed as mean±SD.

	MDA (nmol/g tissue)	**GSH (nmol/mg proteins)**	**GPx (µmol GSH/mg proteins)**
	Testis	Testis	Testis
Control	0.015±0.001^{c}	0.206±0.005^{ab}	0.067±0.007^{a}
Cu	0.018±0.002^{a}	0.066±0.001^{e}	0.026±0.002^{d}
F	0.015±0.005^{c}	0.209±0.001^{a}	0.071±0.001^{a}
L	0.015±0.002^{c}	0.203±0.001^{b}	0.070±0.009^{a}
CuF	0.016±0.002^{b}	0.110±0.001^{d}	0.057±0.008^{b}
CuL	0.015±0.002^{c}	0.179±0.002^{c}	0.035±0.008^{c}

Means that do not share the same letter are significantly different at $p<0.05$.

Results indicated a no difference in the absolute testicular weights of rats exposed to Cu for one month compared to the control group, which agrees with the finding of Chattopadhyay et al. [22]. Other researchers showed a slight decrease of rat testicular weight, in addition to a degenerative and necrotic effect of Cu on the seminiferous tubules and loss of spermatid from the center of the tubules [23]. In humans, Cu can produce adverse effects, including prostate enlargement, prostate infections, erectile dysfunction, depression, anxiety, testicular pain, and testicular cancer [1].

In this study, Cu was associated with lower testosterone concentration in rats; this result is in line with previous research showing that overdose of copper may affect the pituitary receptors, causing a reduction in the concentrations of LH and FSH, the key hormones in controlling testosterone release [24]. As a copper an antagonist of zinc, high copper levels can reduce zinc concentration; notably, zinc plays an important role in the formation of androgens [25]. The accumulation of heavy metals in the testis may lead to the inhibition of steroidogenesis [26].

In this investigation, rats exposed to copper had poorer sperm motility, velocity (VCL, VSL, and VAP), lateral head displacement, beat cross frequency, and vitality. This suggest that accumulation of copper in the epididymis, prostate, and seminal vesicle may inhibit sperm motility, BCF, and ALH, possibly by affecting the spermatozoa energy source, and by modifying the sperm tail shape, which can explain the decrease of the velocity measures VCL, VSL, and VAP. Moreover, Cu ions may accumulate in sperm mitochondria due to attraction to sulfhydryl groups, causing less ATP production, which can reduce sperm motility [27,28]. A high copper level in the reproductive organs is probably responsible for lowering the pH of seminal plasma, which was reported to

decrease sperm motility and the percentage of live sperm [13]. Other authors have found that a pH between 6.2 and 5.2 decreased sperm concentration, movement, and velocity measures VCL, VSL and VAP [29] by deregulating the Na/K ATPase activity [30] that blocks the entrance of calcium, which is responsible for improving the amplitude of flagellar beat [13]. Moreover, as copper is an antagonist of zinc, the deficiency of the latter may lead to low sperm concentration. Compared to other cells, spermatozoa contain less cytoplasm. As the latter is where the antioxidants system is found, having less cytoplasm makes spermatozoa susceptible to oxidative stress through lipid peroxidation [31], which affects the acrosome reactivity reactivity, and increases DNA damage [32]. This may explain the increase in dead sperm observed in the copper treated-group.

In the Fenton reaction, the cupric ions Cu+2 is reduced to cuprous Cu+; the latter is able to catalyze the hydroxyl radical, which is highly reactive, and form lipid radicals from fatty acids [12]. In our study, the group treated with Cu showed higher MDA levels, suggesting that copper ions inhibited the antioxidant reactions [33]. Copper as a destructive metal to tissues can cause lipid peroxidation, which can increase the testicular MDA levels [34].

We expected that high levels of copper in different tissues could produce reactive oxygen species (ROS), DNA damage, and lipid peroxidation [35]. In the present study, the observed low levels of GSH and GPx in the Cu exposed rats were likely to be caused by ROS production [36], and by cellular usage of GSH and GPx in the metabolism and detoxification of copper [23]. In addition, GPx is involved in scavenging hydrogen peroxide and lipid peroxidase caused by copper ions [37].

Although the hawthorn has been reported to treat sexual weakness in Noth Africa [38,39], there are not many studies about the effect of this plant on male fertility. The positive control of L showed higher sperm concentration and VCL in rats after a one-month treatment. As a powerful antioxidant, the fruits of hawthorn (Crataegus spp) may protect Sertoli cells from oxidative stress and may improve sperm quality due to the presence of phenolic compounds, oils, and vitamins. Previous findings showed the existence of vitamin C in the hawthorn and established the role of vitamin C on sperm characteristics [40], and the improvement of sperm concentration in male rabbits [41]. Similarly, our results showed an improvement in sperm quality in the CuL and CuF groups over the Cu group. The vitamin C in Crataegus spp. and in Rosaceae plants in general [40] helps in neutralizing hydroxyl, superoxide, and hydrogen peroxide radicals to prevent sperm agglutination [42]. In addition, vitamin C can both inhibit copper intestinal absorption, and increase its excretion [43]. The ability of vitamin C to scavenge free radicals could explain the remarkable lower levels of MDA observed in theCuL and CuF groups.

Furthermore, the augmentation in sperm motility in the CuL group is in line with the finding of Hu and Xiong [44] who observed that sperm from patients with asthenospermia showed increased

motility when co-incubated with extracts from the genus hawthorn (Crataegus spp). Studies on the chemical composition of C. monogyna revealed the presence of vitamins, flavonoids, and oils [40,45,46] that can act as a source of energy to boost sperm movement, increasing VSL and VAP [46]. This is probably why, in this study, the velocity measures (VCL, VSL, and VAP), ALH, and BCR were higher in the groups administered with C. monogyna extract.

Researchers previously found an effective role of C. monogyna extracts as an antioxidant [47,48] through the scavenging of free radicals and the inhibition of LDL oxidation [49]. Furthermore, the vitamin E in C. monogyna [45] can act as chain-breaking antioxidant [50], and prevents lipid peroxidation and tissue damage [42]. The occurrence of linoleic acid (omega-6); oleic acid (omega-9); oxalic acid bis (trimethylsilyl) ester; palmitic acid; and tetramethyl-cyclodecasiloxane that show powerful antioxidant activity [46] in C. monoyna might explain the lower levels of MDA and the higher levels of both GSH and GPx in the CuF and CuL groups. Previous results showed that omega-6 fatty acids can improve sperm motility [51], which may have contributed to the improvement of VCL, VSL, VAP, ALH and BCF levels in this study.

Quercetin was found in hawthorn, has been reported to act as a growth inhibitor for several malignant tumor cell lines, such as human epididymal cancer [52] by scavenging free radicals and chelating divalent cations [53]. Such a compound might have a role in reducing hydrogen peroxide, increasing sperm antioxidant defenses and preventing DNA damage induced by oxidative stress [54]. Moreover, quercetin may improve sperm quality by preserving the sex organs' functions [55]. Similarly, catechin polyphenols from C. monogyna, on the other side, were shown to reduce ROS by quenching free radicals and chelating transition metals [56], while catechins from green tea were shown to boost reproductive parameters [57]. This may explain the observed improvement of sperm concentration, motility, live sperm, velocity, ALH and BCF in the present study, especially in the CuL group.

In conclusion, copper induced oxidative stress, affecting testicular MDA, GSH, and GPx levels, and sperm quality parameters. The co-administration of extracts of C. monogyna fruits and leaves kept oxidative stress markers to almost their normal physiological ranges and improved semen quality, perhaps by mitigating the copper toxicity.

ACKNOWLEDGEMENTS

Authors would like to thank The General Directorate of Scientific Research and Technological Development (DGRSDT) for financial support (Award number 06/2016, recipient C. ABDENNOUR). Thanks are also given to Pasteur Institute (Algiers) for providing rats.

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International Journal of Minor Fruits, Medicinal and Aromatic Plants. Vol. 6 (2) : 01- 07, December 2020

The aqueous extract of fruits and leaves of Crateagus monogyna Jacq. in mitigating copper sulphate-induced hepatotoxicity and nephrotoxicity of Wistar rats

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Received : 17.08.20 ; Revised : 19.09.20 ; Accepted: 23.09.20

ABSTARCT

This study explores the promising mitigating activity of fruits (F) and leaves (L) aqueous extract of Crateagus monogyna Jacq. (Fam. Rosaceae) against hepatotoxicity and nephrotoxicity induced by copper sulphate (Cu). Adult male Wistar rats were divided into the control (C), two positive controls ssupplemented with F (1.5g/kg bw/day) and L (1.5g/kg bw/day) aqueous extract, Cu group (100 mg/kg bw/day),and two other combined groups having the same dosage (Cu+F, Cu+L). The C. monogyna aqueous extracts and copper sulphate were administrated orally for 30 consecutive days, where liver and kidney glutathione (GSH), malondialdehyde (MDA) and glutathione peroxidase (GPx) were evaluated alongside plasma aspartate aminotransaminase(AST), alanine aminotransaminase(ALT),alkaline phosphatase (ALP), urea and creatinine levels. A significant increase in the activity of AST, ALT and ALP and the creatinine level of the Cu group were observed compared to the control, but Cu+F and Cu+L have significantly decreased AST, ALT,ALP, creatinine and urea levels compared to the Cu group. Cu group has respectively increased hepatic MDA concentration, and decreased GSH level and GP x activity compared to the control. The combined treatments (Cu+F and Cu+L)showed a significant decline in MDA concentration, accompanied with significant raise of GSH and GPx levels compared to the Cu group, as well as both positive controls (F and L) demonstrated a significant augmentations of GSH and GPx levels compared to the control. In kidney, Cu group has respectively increased and decreased MDA concentration and GPx activity, but Cu+F and Cu+L have significantly reduced the MDA concentration and raised both GSH level and GPx activity. To conclude, Cu administration to rats has induced hepatotoxicity and nephrotoxicity, while the combination of this metal with the hawthorn aqueous extract have attenuated such toxicity.

***Keywords*:** Copper,Crateagus monogyna, oxidative stress, rat, toxicity.

INTRODUCTION

Copper is an essential trace element for many biological function; it can play a role as an antioxidant enzyme, and as a cofactor in ceruloplasmin molecule (Sharma *et al*., 2005). Copper is found in animals, plants and microorganisms and even in environmental components as water, atmosphere and soils (Stern *et al*., 2007). Moreover, copper enters in many human activities and it is used in industrial, agricultural and medicinal purposes. This huge usage of copper has increased its dispersion in the environment creating a chronic exposure to living organisms and the whole ecosystems.

In the body, copper enters through the digestive tract, respiration, and it even crosses the skin, where nearly 30-50% is absorbed to the blood stream (Turnlund *et al*., 1997), in which the big portion binds to serum albumin (Anant *et al*., 2018). Copper is located principally in the liver, where its metabolism takes place and it mainly excreted by the bile duct. Copper was postulated to activate the Fenton reaction to form reactive hydroxyl radicals leading to cellular damage (Baureder *et al*., 2012). Thus, excess of copper may provoke the deactivation of ATP7B, which might leads to liver, brain and other organs' injuries such as liver cirrhosis, neurological disorders, kidney malfunctions and red blood destruction, known as Wilson's disease (Allen *et al*., 2006). Long term exposure of rats to copper leads to its accumulation in liver, followed by kidney and brain, in which free tissue copper was positively correlated with oxidative stress and organs' dysfunction (Kumar *et al*., 2016). Liver is known to play a key role in maintaining Cu

homeostasis, and also the xenobiotics' detoxification (Chiang, 2014), since all absorbed nutrients pass into the liver by the portal vein.

Through years, the usages of plants as food, and as medical remedies in treating many diseases such as cancer and diabetes were proven by many researchers (Bhowmik, 2019), where, these plants are cheap and available (Momin *et al*., 2018). Hawthorn was demonstrated to have a beneficial effect on human health (Çoklar *et al*., 2018). It is a spontaneous tree of rosaceous family distributed mainly in Africa, Asia, America and Europe (Muradoglu *et al*., 2019). Hawthorn is being used as a food or a as medicine to treat cardiovascular disorders, stomach troubles, inflammation (Zhang *et al*., 2002), atherosclerotic diseases (Chang *et al*., 2002), respiratory impairments (Arrieta *et al*., 2010) and as an antioxidant (Osawa, 1994). Crataegus monogyna was reported to contain iron, zinc, manganese, magnesium (Özcan *et al*., 2005), in addition to oxalic acid, malonic acid, palmitic acid, oleic acid, linoleic acid, and other essential oils (Bechkri *et al*., 2017). As a result, the presence of flavonoids, chlorogenic acid and Triterpenes make it a powerful antioxidant useful to many medicinal treatments (Nabavi *et al*., 2015).

The present work explores the possible mitigating activity of Crataegus monogyna fruits and leaves aqueous extracts against the chronic toxicity of copper through the evaluation of hepatic and renal markers of male Wistar rat.

MATERIAL AND METHODS

Plant and copper preparation

The common hawthorn trees Crataegus monogynaare grown spontaneously along the northern zone of Algeria, exceeding 3 meters in length, and characterized by green leaves, white flowers and red fruits; the latter reaches maturity in mid-autumn. The plant authentication was made by the staff of the Department of Biology, in which voucher specimen were deposited in the laboratory. Fruits and leaves of C. monogyna were harvested freshly in November from Annaba area, northeast Algeria. Therefore, fruits and leaves were separately weighted daily, crushed in 20 ml of distilled water and were kept overnight (12 hours) at room temperature. Then, the homogenates were filtered in the morning to obtain the aqueous extracts of F and L, where rats receive the equivalent of 1.5g/kg bw/day of the filtered extract obtained. 100 mg of copper sulfate pentahydrate salt ($CuSO4{\cdot}5H2O$) was dissolved daily in distilled water. The combined treatments were made by mixing volumes of dissolved copper sulphate and the filtered aqueous extracts (Cu+F, Cu+L), which immediately administered to animals by gavage; this procedure is repeated every day during 30 days.

Experimental design

Wistar rats were purchased from the Pasteur institute, Algiers (Algeria) weighing 196±8 g that received tap water and standard diet ad libitum. Thirty-six males were divided equally into 6 groups; the control (C) having a standard diet, the copper (Cu: 100 mg/Kg bw), the fruits(F:1.5 g fruits/kg bw), the leaves (L: 1.5 g leaves/kg bw), the Cu+F(100 mg Cu sulphate/Kg bw + 1.5 g fruits/kg bw) and the Cu+L(100 mg Cu sulphate/Kg bw + 1.5 g leaves/ kg bw) group. Rats were sacrificed by decapitation after 30 consecutive days of oral administration of copper solution and fruits and leaves aqueous extracts. Blood was received in heparinized test tubes, was immediately centrifuged at 3000 rpm for 10minutes, and then the plasma was stored at -20 °C together with the liver and the kidney till further analysis. Animals' experiments were authorized by the Ethical Committee of Animal Sciences at the University of Badji Mokhtar-Annaba.

Plasma markers assay

The dosage of asparatateaminotransaminase (AST), alanine aminotransferase(ALT), alkaline phosphatase (ALP),urea and creatinine have been carried out by the colorimetric method according to the technical data sheet of the SpinreactKit,Spain.

AST catalyses the transfer of an aspartate moiety to alpha-ketoglutarate to form glutamate and oxaloacetate, the latter is reduced to malate in the presence of dehydrogenated (MDH) and NADH (Murray, 1984).

In the ALTreaction, an amino group from alanine is transferred to α-ketoglutarate forming glutamate and pyruvate thatis reduced to lactate by lactate dehydrogenase (LDH) and NADH (Murray, 1984).

Fig. 1: Fruits and Leaves of Crateagus monogyna Jacq. collected in 2018.

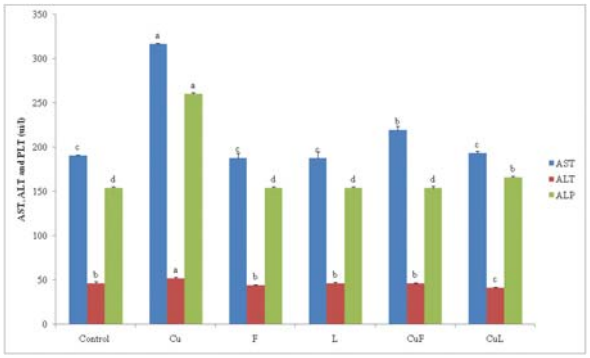

Fig. 2: Plasma variation (mean±SD) of AST, ALT and ALP activity) levels of rats treated by copper, C. monogyna and the combination of copper and hawthorn for 30 days. Means that do not share the same letter are significantly different at p<0.05.

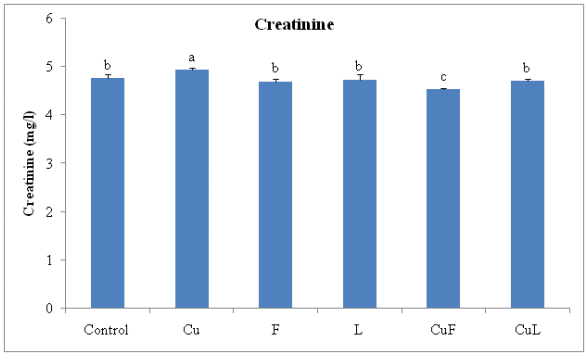

Fig. 4: Plasmavariation (mean±SD) of creatinine concentration of rats treated by copper, *C. monogyna* and the combination of copper and hawthorn for 30 days.Means that do not share the same letter are significantly different at p<0.05.Means that do not share the same letter are significantly different at p<0.05.

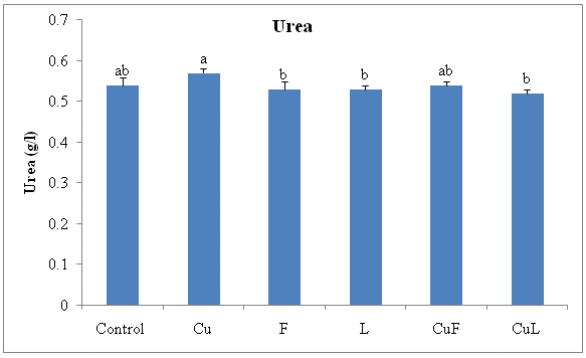

Fig. 3: Plasma variation (mean±SD) of urea concentration of rats treated by copper, *C. monogyna* and the combination of copper and hawthorn for 30 days. Means that do not share the same letter are significantly different at p<0.05.

ALP catalyses the transfer of the phosphate groupfrom p-nitrophenylphosphate (pNPP) to 2-amino-methyl-1 propanol by releasing p-nitrophenol and phosphate (Wenger *et al*., 1984; Rosalki *et al*., 1993).

Urea in the sample is hydrolyzed enzymatically into ammonia (NH4+) and carbon dioxide (CO2). Ammonia ions formed reacts with salicylate and hypochlorite (NaClO), in presence of the catalyst nitroprusside, to form a green indophenol sample (Kaplan, 1984).

Creatinine reacts with alkaline picrate forming a red complex. The intensity of the color formed is proportional to the creatinine concentration in the sample (Murray *et al*., 1984).

Oxidative stress assay

100mg of frozen liver and kidney were thawed and transferred to test tubes for the determination of glutathione reduced (Weckbecker and Cory, 1988), total proteins by using the Coomassie Brilliant Blue G-250 (Bradford, 1976), malondialdehyde (Ohkawa *et al*., 1979), and glutathione peroxidase (Flohe and Günzler, 1984).

Statistics

Statistics was appliedby using ANOVA followed by Tukey test (MINITAB 18 Software). Results are expressed as mean ±standard deviation. The significant test was considered at $p<0.05$.

RESULTS AND DISCUSSION

Copper intake has significantly increased the AST and the ALT activity compared to the control, meanwhile these enzymes were significantly decreased in the CuF and CuL groups compared to the Cu-treated group, while the positive control F and L have maintained the same activity of AST and ALT as that of the control (Figure 2).

The ALP activity of the Cu group showed a significant increase when compared to the control, but it decreased significantly in the CuF and CuL groups compared to the Cu group (Figure 2).

Liver enzymes are usually used to detect the hepatocytes' damage. In this study, the increase of plasma AST, ALT and ALP activities was noticed in animals intoxicated with copper for thirty days, such result is in line with that reported by Emad and Shimaa, (2016) in serum rats, and with that of urinary AST of male rats after the treatment with 140 mg Cu/kg/day of copper (Anonymous, 1993). Other previous studies have confirmed that copper sulphate may lead to liver cancer (Tchounwou *et al*., 2008), which probably originated from the deficiency of zinc and vitamin B6 during copper intoxication (Eck and Wilson, 1989). Moreover, Cu supplementation that induced liver cirrhosis and hepatocytes necrosis (Winge and Mehra, 1990) agrees with the augmentation of enzymes' activity observed in this study. Cell injury following copper accumulation was reported to be caused by ROS generation (Manzl *et al*., 2004; Emad and Shimaa, 2016), where a positive correlation was confirmed by liver free copper concentration and organ dysfunction (Kumar *et al*., 2016) in rats supplemented with 100 and 200mg/Kg bw/day. In contrast, Wistar rats exposed to copper (60mg Cu/kg bw) for four weeks and also young people (0.315 mg Cu/kg/day) for nine months did no demonstrate any variations in serum ALT and AST activities (Galhardi *et al*., 2004; Zietz *et al*., 2003).

Urea level has a slight increase in rats exposed to Cu compared to the control, but it decreased significantly in the CuL group compared to the Cu group (Figure 3).

Results of creatinine concentration of rats exposed to Cu toxicity demonstrated a significant increase compared to the control, but it decreased significantly in the CuL and CuFgroups compared to the Cu group (figure 4).

Plasma urea and creatinine concentration are used to follow the kidney excretion status. Therefore, urea level has not been affected in animals exposed to copper for one month, while creatinine showed a remarkable increase, indicating the possible copper toxic effect. Alongside, urea plasma level was increased in rats' experienced copper intoxication (Akomolafe *et al*., 2016), as well as the observed relationships between Cu exposure and human renal disorders (Sinkovic *et al*., 2008). Furthermore, blood urea nitrogen level was clearly correlated with kidney free copper content in rats exposed to copper for 90 days (Kumar *et al*., 2015). Contrary, 50 µmol/kg bw of inorganic copper for 30 days have not made any observed changes in the concentration of blood urea and creatinine in Wistar rats (Abou-seif *et al*., 2003).

Hepatic MDA level demonstrated a significant increase in Cu-exposed group compared to the control, while the positive controls of C. monogyna F and L extracts have almost the same level as that of the control.

On the other hand, hepatic GSH concentration and GPx activity were decreased significantly by Cu treatment compared to the control, with a remarkable increase in the combined treatment CuF and CuL compared to the Cu group. However, the positive controls of F and L have showed a

significant rise in the levels of GSH and GPx compared to the control.

Renal MDA concentration was increased significantly on the Cu group compared to the control, although it has been decreased significantly in rats of the CuF and CuL groups compared to the Cu group. The positive groups F and L were not significantly different than that of the control.

Renal GSH level and GPx activity have been decreased significantly in the Cu group compared to the control, but when compared to the Cu group, the two markers of CuF and CuL groups were highly significant. Interestingly, the positive group F of hawthorn extracts hashigher GSH and GPx levels than that of the control.

High copper content in cells could create an imbalance between oxidative stress production and the antioxidant defense system (Sies, 2015), as that of Haber-Weis is reaction, which can be activated by metal toxicity to generate reactive oxygen species (Rosario *et al*., 2017), leading to mitochondrial dysfunction (Myers *et al*., 1993). As a result, copper may provoke cell membrane damages through the augmentation of lipid peroxidation that could lead to liver and kidney cells' necrosis, which might be explained by the increase of hepatic and renal MDA concentration in rats administrated with copper sulphate during four consecutive weeks. Such result is in-line with that of Kumar *et al*. (2016) who demonstrated an augmentation of hepatic and renal MDA level in parallel with the tissue free copper after exposure of rats to copper sulphate during three months.

The remarkable decrease of hepatic and renal GSH and GPx levels after one month exposure to copper is likely affected by ROS generated by copper ions, exactly as what was reported previously, where GSH concentration was decreased in rat after copper sulphate injection (Ossola *et al*., 1997), and also the observed decrease in hepatic GSH level and GPx activity after Cu overload in rats (Rosario *et al*., 2017). Thus, copper ions were demonstrated to decrease GSH concentration due the high affinity of sulfhydryl group to this metal (Rosario *et al*., 2017), and also glutathione concentration of liver was inversely correlated with serum aminotransaminases and tissue free copper of rats intoxicated with copper (100 and 200mg/Kg bw) during 90 days (Kumar *et al*., 2016). Certainly, copper ions accumulated in liver and kidney during this experiment are highly attracted to sulfhydryl groups, which leads to lowering the level of glutathione and by consequent induces a decrease in GPx activity. Indeed, copper ions accumulated in liver and kidney of the exposed rats are highly attracted to sulfhydryl groups, which leads to lowering the level of glutathione and by consequent induce a decrease in GPx activity (Rosario et al., 2017).

From the data obtained in this investigation, the previous markers of rats supplemented with the combined treatment of copper and hawthorn were almost within the normal physiological ranges, that to see C. monogyna fruits and leaves extracts have the capability to mitigate the disturbing action of copper ions. The likely reason is that C. monogyna is rich in many components as vitamin C and phenols (Muradoglu *et al*., 2019), probably this is why it is used as an antioxidant to treat certain diseases (Osawa, 1994). This could explain the role played by C. monogyna in strengthening the antioxidant system through the augmentation of hepatic and renal GSH and GPx levels. Accordingly, supplementing Crataegus pinnatifida leaves extract to rats have demonstrated a significant increase in antioxidant enzymes (Wang *et al*., 2011). Moreover, it was found that the presence of linoleic, oleic and palmitic acids in C. monogyna could enhance the antioxidant activity (Bechkri *et al*., 2017), and also may reduce copper intestinal absorption since hawthorn was noted to be rich in vitamins, zinc, and iron, which might antagonist copper ions intake (Ozcan *et al*., 2005). Therefore, the mitigating benefit of hawthorn is likely established by scavenging the superoxide anions, hydroxyl radicals, hydrogen peroxides and reducing lipid peroxidation (Rice-Evans, 2004).

CONCLUSION

The aqueous extract of fruits and leaves at the dose of 1.5 g/kg bw administered orally to Wistar rats have proved to have mitigating activity towards

liver and kidney markers' disturbances induced by copper for thirty days.

ACKNAWLEDGMENT

Authors would like to thank The General Directorate of Scientific Research and Technological Development (DGRSDT) for financial support (Award number 06/2016, recipient C. ABDENNOUR). Thanks are also given to Pasteur Institute (Algiers) for providing rats.

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International Journal of Minor Fruits, Medicinal and Aromatic Plants. Vol. 7 (1) : 19-25, June 2021

The protective effect of Crataegus monogyna Jacq aqueous extract (fruits and leaves) on blood cells and lipid profile of rats after copper induced-toxicity

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Received : 21.02.2021 ; Revised : 12.03.2021 ; Accepted : 15.03.2021

ABSTRACT

The objective of this work is to use the Hawthorn Crataegus monogyna, as a protective agent against copper chronic intoxication. Male Wistar rats were divided into six groups; the control received tap water, standard diet ad libitum, two positive controls treated respectively with Hawthorn leaves and fruits aqueous extract, a group treated with Cu and finally, two groups treated with Cu+leaves (CuL) and Cu+fruits (CuF). The treatment was done by gavage for 30 consecutive days, where: glucose-6-phosphate of erythrocytes (G6PD), white blood cells (WBC), red blood cells (RBC), platelets (PLT), hemoglobin (HGB), hematocrits (HCT), mean corpuscular volume (MCV), triglycerides (TRIG), cholesterol (CHOL), high density lipoproteins (HDL) and low density lipoproteins (LDL) were measured. Copper treatment reduced G6PD, RBC, HGB, HCT, MCV, TRIG and CHOL levels, compared to the control. Compared to the Cu group, the two combined treatments (Cu L and Cu F) have an increase on G6PDH, RBC, HGB, HCT, MCV, TRIG and CHOL levels, with a decrease in WBC, PLT, and LDL levels. As a conclusion, hawthorn aqueous extracts have mitigated copper toxicity towards blood cells and LDL of wistar rats.

Keywords **:** Glucose-6-Phosphate Dehydrogenase, hawthorn, high density lipoprotein, red blood cells

INTRODUCTION

Copper is a trace element essential for many biological processes, but it becomes harmful when it exceeds the threshold level (Abbas *et al.,* 2018). About 60% of consumed copper is absorbed in the stomach and the small intestine (DES, 2013), where its absorption, distribution, detoxification and elimination are well controlled (Kumar *et al.,* 2015). Copper homeostasis maintains of copper distribution and prevents causing any negative effects to cellular defense system (Quamar *et al.,* 2019). However, both augmentation and deficiency of copper concentration may cause physiological disorders (Chambers *et al.,* 2010). Thus, increases in copper concentration in body have been reported to be associated with many pathological conditions (Parmar *et al.,* 2002; Ozcelik and Uzun, 2009) as anemia by the red blood destruction (DES, 2013), abnormal lipid profile (Burkhead and Lutsenko, 2013) and lower triglycerides concentrations (Wuolikainen *et al.,* 2014). Furthermore, high copper level provokes cell injury (Saravu *et al.,* 2007) by oxidizing cell membranes (James *et al.,* 1999; Saravu *et al.,* 2007), mitochondrial dysfunction and lowering antioxidant enzymes, leading to oxidative stress damage (Tiwari *et al.,* 2018).

Through the years, interest of using plant compounds has been growing faster in worldwide due to their benefits on health (Nandi and Ghosh, 2016). Hawthorn, *Crataegus monogyna,* is one of very common shrub plant used in medicinal treatments (Fong and Bauman, 2002), which considered a relatively safe herb and without serious adverse effects (Zapfe, 2001). The plant is well distributed in the Mediterranean region. *C. monogyna* is rich in proanthocyanidins and flavonoids (Bahorun *et al.,* 1996), which are superoxide anion (Keser *et al.,* 2014), hydroxyl radical, hydrogen peroxides scavengers and lipid peroxidase reducer (Bahorun *et al.,* 1994 ; Rice-Evans, 2004), which make it a powerful antioxidant (Yao *et al.,* 2008). Interestingly, flavonoids of Hawthorn have the ability to inhibit copper intake (Kuo *et al.,* 1998).

The aim of this study is to investigate the ability of the common *C.monogyna aqueous extract* of both fruits and leaves in protecting blood

biomarkers and lipid profile of Wistar rat intoxicated with copper sulfate.

MATERIALS AND METHODS

Plant and preparation

Crataegus monogyna is grown spontaneously along the Algeria northern zone, exceeding 3 meters in length, and characterized by green leaves, white flowers and red fruits; the latter reaches maturity in mid-autumn. Fruits and leaves were harvested freshly in November from Annaba area, northeastern Algeria. 1.5g/kg bwe of fruits (F) and leaves (L) were weighted daily, crushed in an appropriate volume of distilled water (where each rat takes 1ml of the obtained extract) and were kept overnight at room temperature. The two homogenates were filtered in the morning for obtaining the of F and L *aqueous extract*. Copper sulfate powder was dissolved daily directly before carrying out the tests in distilled water. The mixture of copper + F and copper + L where prepared daily using the same doses.

Experimental design

Wistar rats were purchased from the Pasteur institute, Algiers (Algeria) weighing 196±8 g, that received tap water and standard diet *ad libitum*. Thirty-six males were divided equally into 6 groups; the control (C) having a standard diet, the copper (Cu: 100 mg $CuSO_4.5H_2O$/Kg bw), the fruits (F:1.5 g fruits/kg bw), the leaves (L: 1.5 g leaves/ kg bw), the Cu+ F (100 mg $CuSO_4.5H_2O$ /Kg bw + 1.5 g fruits/kg bw) and the Cu+ L (100 mg $CuSO_4.5H_2O$ /Kg bw + 1.5 g leaves/kg bw) group. Rats were sacrificed by decapitation after 30 consecutive days of copper oral administration, fruits and leaves solutions. Blood was collected in heparinized and EDTA test tubes, in which heparinized tubes were centrifuged at 3000 rpm for 10 minutes, and then the plasma was stored at -20°C till further analysis. Animals' experiments were authorized by the Ethical Committee of Animal Sciences at the University at the Badji Mokhtar university of Annaba (Algeria).

Erythrocytes G6PD assay

Glucose-6-phosphate deshydrogenase (G6PD) dosage was measured using Mindray BS-380 apparatus, according to BIOLABO REAGENT (U.V Kineticmethod) kit and the reaction scheme (Beutler *et al.,* 1977). The rate of increase in NADPH concentration measured at 340 nm is proportional to the G6PD activity of the specimen.

Complete blood count

The complete blood count was realized by using the blood counter Abacus 4.

Triglycerides assay

Triglyceride has been assayed using the enzymatic colorimetric method; according to the technical user manual of the Spinreact Kit (Spain). The triglycerides incubated with lipoprotein lipase (LPL) release the glycerol and free fatty acids. Glycerol is converted to glycerol-3-phosphat (G3P) and adenosine-5-diphosphate (ADP), by the glycerol kinase and adenosine phosphate (ATP). The G3P is then converted by the glycerol phosphate dehydrogenase (GPO) in active ingredient to dihydroxyacetone phosphate (DAP) and hydrogen peroxidase (H_2O_2). The latter reacts with 4-aminophenazone (4-AP) and p-chlorophenol in the presence of peroxidase (POD) to give a red color (Bucolo and David 1973).

HDL Cholesterol assay

The dosage of high density lipoproteins HDL has been carried out by the enzymatic method (Spinreact Kit, Spain).The very low density (VLDL) and low density (LDL) lipoproteins were precipitated by phosphotungstate in the presence of magnesium ions. After centrifugation the supernatant contains high density lipoproteins (HDL). The HDL cholesterol fraction was determined using the total cholesterol enzymatic reagent (Naito, 1984; Grove, 1979).

LDL cholesterol assay

The dosage of low density lipoproteins (LDL) was assayed according to technical guide of Spinreact Kit, Spain. Direct determination of serum LDL (low-density lipoprotein cholesterol) levels was carried out without the need for any sample pre-treatment or centrifugation of the sample (Friedewald *et al*., 1972).

Cholesterol assay

The assay of high density lipoproteins (HDL) was realized by the enzymatic method according

to the technical data sheet of the Spinreact Kit, Spain (Naito, 1984).

RESULTS AND DISCUSSION

Hematological markers are presented in table 1 showed a significant decrease in Cu group in G6PD, RBC, HGB and HCT levels, contrary Cu has augmented the WBC, PLT and MCV levels compared to the control. No change in the group treated with Cu F, while Cu L group showed a significant increase in G6PD, RBC, HCT and MCV levels, and a significant decrease in WBC level compared to the control.

Table 2 represents the rat's lipid profile exposed to copper for one month. Our Results showed significant decreases in triglyceride and cholesterol, while HDL and LDL levels kept the same levels compared to the control. Cu F showed an augmentation in triglyceride, cholesterol and decreased the HDL and LDL levels compared to the Cu group. In the group treated with Cu L, triglyceride, cholesterol and HDL levels augmented significantly, while no change was observed in LDL level compared to the Cu group.

In this research, copper sulfate administrated to rat for one month decreased significantly the G6PD, RBC, HGB and HCT levels, while it increased WBC, PLT and MCV. Recently, high copper level decreased the RBC counts and HGB concentration in rats (Akomolafe *et al.,* 2014) as a result of erythrocytes hemolysis induced by the free copper ions. The low activity of G6PD in rats of the copper group might be related to the inhibition of the enzyme by copper ions, an enzyme responsible of the red cells protection from oxidative stress by maintaining the GSH level through NADPH generation (Joshi *et al.,* 2002). Moreover, toxic copper was reported to induce hemolysis, leading to red blood dysfunction (Savaru *et al.,* 2007), and disturbs the erythropoiesis by affecting iron metabolism in the intestinal tracts, where copper and iron are antagonists (Pmila *et al.,* 1991). The observed iron deficiency during high copper level has led to anemia (Eck and Wilson, 1989) and methaemoglobinaemia (Oldenquist and Salem, 1999; Ahasan *et al.,* 1994), which confirm that copper is involved in the erythropoiesis process (Samanta *et al.,* 2011). On the other hand, the MCV

Table 1: Mean ± SD of some hematological markers in the different groups after treatments by copper sulphate and *C. monogyna* for one month.

	Control	Cu	F	L	Cu F	Cu L
G6PD (mUI/10^9)	121.4 ± 0.9^{b}	83.3 ± 0.6^{d}	124.1 ± 0.8^{a}	121.1 ± 2^{b}	121 ± 0.6^{b}	101 ± 0.4^{c}
WBC (10^3/mm)	7.49 ± 0.35^{c}	12.04 ± 0.1^{a}	7.95 ± 0.8^{c}	7.87 ± 0.7^{c}	8.01 ± 0.9^{c}	9.87 ± 0.4^{b}
RBC (10^6/mm^3)	10.36 ± 0.3^{a}	8.22 ± 0.2^{e}	9.86 ± 0.4^{bc}	9.47 ± 0.2^{cd}	10.05 ± 0.03^{ab}	9.017 ± 0.06^{d}
PLT (10^3/mm^3)	317 ± 1.7^{c}	899 ± 2.4^{a}	316 ± 20.9^{c}	307 ± 8.1^{c}	305 ± 5.8^{c}	435 ± 30.4^{b}
HGB(g/L)	151.8 ± 5.04^{a}	133.8 ± 3.4^{c}	152 ± 3.6^{a}	152 ± 2.1^{a}	152 ± 0.8^{a}	144.6 ± 0.8^{b}
HCT (%)	51.04 ± 0.8^{b}	40.7 ± 0.7^{d}	52.4 ± 0.8^{a}	50.7 ± 0.7^{b}	50.4 ± 0.5^{b}	47.3 ± 0.8^{c}
MCV (fl)	51.6 ± 0.5^{a}	39.3 ± 0.8^{c}	51.1 ± 0.7^{a}	51 ± 0.6^{a}	51 ± 0.6^{a}	47.8 ± 0.7^{b}

Means that do not share the same letter are significantly different ($p<0.05$), according to one-way ANOVA, followed by Tukey test. G6PD: 6-phosphate; WBC: white blood cells; RBC: red blood cells; PLT: platelets, HGB: hemoglobin; HCT: hematocrits; MCV: mean corpuscular volume.

Table 2: Mean ± SD of Biochemical markers in the different groups after treatments by copper sulphate and *C. monogyna* for one month.

	Control	Cu	F	L	Cu F	Cu L
TRIG (g/l)	0.88 ± 0.07^{a}	0.13 ± 0.03^{d}	0.56 ± 0.02^{c}	0.63 ± 0.03^{b}	0.55 ± 0.03^{c}	0.55 ± 0.03^{c}
CHOL (g/l)	0.68 ± 0.007^{a}	0.21 ± 0.01^{c}	0.46 ± 0.01^{c}	0.53 ± 0.02^{b}	0.54 ± 0.02^{b}	0.55 ± 0.01^{b}
HDL (g/l)	0.31 ± 0.01^{e}	0.31 ± 0.01^{e}	0.56 ± 0.008^{a}	0.45 ± 0.007^{b}	0.37 ± 0.01^{c}	0.33 ± 0.008^{d}
LDL (g/l)	0.18 ± 0.008^{a}	0.17 ± 0.01^{ab}	0.10 ± 0.005^{c}	0.09 ± 0.001^{c}	0.11 ± 0.01^{c}	0.15 ± 0.01^{b}

Means that do not share the same letter are significantly different ($p<0.05$), according to one-way ANOVA, followed by Tukey test. TRIG: triglycerides; CHOL: cholesterol, HDL: high density lipoproteins; LDL: low density lipoproteins.

and HCT level have increased significantly when rats exposed to copper, without affecting RBC count and HBG concentration (Akomolafe *et al.,* 2016). Liver injury by the copper toxicos is may lead to coagulation cascade (Nelson, 2002); this perhaps explains the observed rise in PLT levels in our finding, which was not the case in the study of Ganong, (2009) who reported that high copper charge had decreased the PLT level by inhibiting the thrombopoietin production. Copper may cause inflammatory reactions to some organs such as liver, heart and kidneys, which may explain the increase of WBC as the macrophages that are sensitive to heavy metals toxicity (Witeska and Wakulska, 2007).

The combined treatment of copper and hawthorn fruits extract in this study showed an increase in G6PD activity and HCT levels, without affecting the other parameters. Thus, *C. monogyna* seems to play an important role in free radicals scavenging induced by copper sulphate, as the study of Bernatonienë *et al.* (2008), who indicated that aqueous and ethanolic extracts have the capacity in protecting cells from oxidative stress. Moreover, hawthorn was reported to be rich in polyphenols (Liu *et al.,* 2019), that have protective activity for hematological markers against lead toxicity (Aksu *et al*., 2012). Also, the active compounds in *C. monogyna* seem to have the ability to enhance the antioxidant system by rising G6PD activity to protect red blood cells against stress injuries. This enzyme is the main supplier of protons through the coenzyme NADP to generate reduced glutathione.

The remarkable triglycerides and cholesterol concentrations decrease in rats having toxic copper dose after thirty days consecutive exposure were in conformity with the studies of Mondal *et al.,* (2007) and Babaknejad *et al.,* 2015). The maintaining level of LDL and HDL in this investigation was probably linked to the HDL synthesis from LDL via the modulation of HMG-CoA reductase activity by copper (Mondal *et al.,* 2007). Contrary, copper administration to cows (40mg/kg) had led to a cholesterol concentration increase (Engle *et al.,* 2001) and cholesterol and LDL in rats as results of the oxidative stress (Galhardi *et al.,* 2004).

The *C. monogyna* administration in both L and F groups has reduced the triglycerides levels, cholesterol, and LDL, while it raised the HDL production. In fact hawthorn given to rats at 2% of the diet was demonstrated to have a hypocholesterolemic and vasoprotective activities (Kwok *et al.,* 2010). Researchers found that the alcoholic extract of the *C. monogyna* berries lowered significantly the cholesterol, triglycerides and the LDL levels (Kausar *et al.,* 2011). Furthermore, *C. monogyna* could increase the receptors capacity to bind to LDL and therefore prevent the cholesterol augmentation (Kausar *et al.,* 2011) and enhancing the cholesterol elimination to bile (Rajendran *et al.,* 1996). Hawthorn was also been found to decrease the serum levels of cholesterol, LDL-cholesterol, and triglycerides in hypercholesterolemic and atherosclerotic animals (Chang *et al.,* 2002). Also studies showed that hawthorn may lower the body weight as our results indicated, and it used to treat obesity and weight control (Kausar *et al.,* 2012).

In the combined group Cu L and Cu F, the HDL level increased significantly, which means that the hawthorn has a beneficial effect, explained by the presence of catalytic metal ions, that increase the long and short chain cholesterol ester and phospholipids (Abuja and Albertini 2001). While LDL decreased significantly particularly HDL with high copper concentration perhaps by accelerating the LDL oxidation (Raveh *et al.,* 2001).

CONCLUSION

The copper induced rat toxicity during thirty days has disturbed most blood parameters and lipid profile, while the co-administration of *C. monogyna* leaves and fruits extracts has led to a mitigating effect by normalizing many blood biomarkers.

ACKNOWLEDGMENT

Authors would like to thank The General Directorate of Scientific Research and Technological Development (DGRSDT) for financial support.

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ATTESTATION
DE PARTICIPATION

Le Président de l'AT-BVBR, atteste que

REMITA FERIEL

a présenté au VI[ème] congrès international de Biotechnologie et Valorisation des Bio-Ressources,

organisé par l'AT-BVBR du 20 au 23 Mars 2018 à Tabarka - Tunisie,

une communication par Affiche intitulée

C. AFFICHE N°:487

Activity of Hawthorn on hematological markers of Wistar rat under copper intoxication

REMITA FERIEL , CHERIF ABDENNOUR

Président de l'AT-BVBR
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SNBES 2018
08-09 Octobre 2018

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Je soussignée Dr. Slimani S. , présidente du Séminaire National Biologie Environnement et Santé SNBES 2018, atteste que: **REMITA Feriel** a présenté : **une communication orale**

Intitulée : «ACTIVITY OF HAWTHORN ON REPRODUCTIVE MARKERS OF WISTAR RAT UNDER COPPER INTOXICATION»

Co-Auteurs: Cherif ABDENNOUR

La présidente du Séminaire:
Dr. Souheila Slimani

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CERTIFICATE OF ATTENDANCE

This is to certify that

Name : REMITA

Surname : Feriel

Participated and presented the communication:

Entitled : Activity of hawthorn on hepatic and renal markers of wister rat under copper intoxication

Authors : Remita feriel, Cherif abdennour, Remita feriel, Cherif abdennour

Type : Poster

at the 30th international congress of the Tunisian Society of Biological Sciences (ATSB) held in Sousse, Tunisia on 25-28 march 2019.

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La Présidente du Congrès et le Président du Comité Scientifique attestent par la présente que :

Mme/Mlle/Mr. **Remita Feriel,** Université Badji Mokhtar, Annaba, Algérie
a participé au **1er Congrès international de Biodiversité, Risques Environnementaux et Santé Publique CIBRESP, En ligne - via Zoom- les 07 et 08 Avril 2021** par une communication par affiche intitulée :
« The activity of hawthorn on hepatic and renal oxidative stress of wistar rat under copper intoxication »
En collaboration avec : **Abdennour Cherif**

Le Président du Comité Scientifique
Pr. Nasri Hichem

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Les 07 et 08 Avril 2021