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Faculty of life and Natural Sciences
Department of Animal Biology

جامعة الإخوة منتوري قسنطينة
كلية علوم الطبيعة و الحياة
قسم بيولوجيا الحيوان

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Presented by: Ibtissam BAGHRICHE

Examination board:

President:	**A. CHETTOUM**	**Prof.**	**University Frères Mentouri Constantine-1**
Supervisor:	**S. ZERIZER**	**Prof.**	**University Frères Mentouri Constantine-1**
Examiners:	**S. DAHAMNA**	**Prof.**	**University Ferhat Abbas Sétif**
	A. ZELLAGUI	**Prof.**	**University of Oume el Bouaghi**
	F. BENCHEIKH	**M.C.A.**	**University Ferhat Abbas Sétif**
	F. TEBBANI	**M.C.A.**	**University Frères Mentouri Constantine-1**

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This thesis is dedicated to my family, my father MOUHAMED and my mother BOUBA who gave me the courage and the support I needed to continue.

To my husband AHCENE, who has been a constant source of support and encouragement during all the hard periods of research and life.

To my daughters: ARWA, LINA and TAKWA, to my little angel: MOUHAMED ELHOUCEINE.

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Table of contents

Table of contents

Table of contents

List of abbreviations

5-FU: 5-Fluorouracil

ADMA: Protein Asymmetric Dimethyl Arginine

ATP: Adenosine triphosphate

BHA: Butylated hydroxyanisole

BHT: Butylated hydroxytoluene

BSA: Bovine Serum Albumin

C6: rat brain tumor cells

CPCSEA: Committee for the Purpose of Control and Supervision of Experiments on the Animal

CVD: Cardiovascular Disease

CβS: β-synthase

DMEM-HG: Dulbecco's modified eagle medium

DMSO: Dimethyl sulfoxide

DPPH˙: 2,2-diphenyl-1-Picrylhydrazyl

DTNB: Dithiobis-2-Nitrobenzoic acid

ECs: Endothelial Cells

ET: Electron Transfer

EtOAc : Ethyl acetate

FBS: Fetal Bovine Serum

FRAP: Ferric Reducing Antioxidant Power

GK: Glycerol Kinase

GPO: Glycerol Phosphate Oxidase

GPx: Glutathione Peroxidase

H2O2: Hydrogen Peroxide

HAT: Hydrogen Atom Transfer

HDL-c: High Density lipoprotein

HeLa: Human Cervix Carcinoma

HHcy: Hyperhomocysteinemia

HNE: 4-Hydroxy-2- Nonenal

IC50: The half inhibitory concentration

LA: Left Atrium

LDL-c: Low Density Lipoprotein

LP: Lipoprotein Lipase

LPL: Lipolytic enzyme

LV: Left Ventricle

MTHF: N-5-methyl-tetrahydrofolate

n- BuOH: n-butanol

NADPH: Nicotinamide Adenine Dinucleotide

NO•: Nitric oxide

O2 •-: Superoxide anion

ON: Oval Nuclei,

ONOOH:Nitroperoxide

ORAC: Oxygen Radical Absorbance Capacity

POD: Dihydroxyacetone phosphate Peroxidase

PVS: Peripheral Vascular System

RA: Right Atrium

RO•: Alkoxyl Radical

ROO•: Peroxyl Radical

ROS: Reactive Oxygen Species

RTCA-SP: Real Time Cell analyzersingle Plate

SAH: S-adenosylhomocysteine

SAM: S-Adenosyl Methionine

SMCs: Smooth Muscle Cells

SPSS: Statistical Package for
Social Science

TBS: Tris-Buffered Saline

TCA: Acide trichloracétique

Tch: Total cholesterol

TEAC: Trolox Equivalent Antioxidant Capacity

TG: Triglycerides

THF: tetrahydrofolate

TNF-α: Tumor Necrosis Factor-α

TRAP: Total Radical Trapping Antioxidant Parameter

UPR: Unfolded Protein Response

VCAM-1: Vascular Cell Adhesion Molecule-1

VEC: Vascular Endothelial Cells

VLDL: Very Low Density Lipoproteins

VSMC: Vascular Smooth Muscle Cells

XDH : Xanthine Dehydrogenase

XO : Xanthine Oxidase

XOR : Xanthine Oxido Reductase

List of Figures

List of Tables

Introduction

Introduction

Butz and du Vigneaud discovered homocysteine (Hcy) in 1932 when they heated methionine in sulfuric acid and got a substance with properties similar to cysteine, which they termed "homocysteine ", because it was a homolog of cysteine (**Tsiami and Obersby, 2017**).

McCully first proposed the homocysteine "hypothesis of arteriosclerosis" in 1969 after observing early atherothrombosis of the peripheral, coronary, and cerebral vasculature in infants with homocystinuria during autopsies (**Kaul *et al*., 2006**).

Homocysteine is a sulfur amino acid. Its metabolism consists of two pathways, remethylation pathway to methionine, which requires folate and vitamin B12, and a transsulfuration pathway to cystathionine, which needs vitamin B6 (**Selhub, 1999**). The main function of Hcy in human bodies is to act as a biochemical intersection between methionine metabolism and the biosynthesis of cysteine, which plays various important roles in human bodies (**Mishra, 2016**).

Hyperhomocysteinemia (HHcy), characterized by an increase in the plasma level of Hcy, is a disorder that causes widespread hazards to human health (**Zhang *et al*., 2005**). HHcy is related with cardiovascular disease, atherosclerosis and reactive oxygen (**Mendes *et al*., 2014**), hepatic lesions and abnormal lipid metabolism (**Latour *et al*., 2015**).

Several researches has found that hyperhomocysteinemia influence on carcinogenesis which is related to low folate levels and other vitamin B deficiencies produced by the exact metabolic mechanisms that cause hyperhomocysteinemia (**Kathpalia *et al*., 2022**).

Cellular defense against reactive oxygen species (ROS) is carried out by intracellular systems, such as antioxidant enzymes or decreasing agents like vitamin C (**Filip *et al*., 2010**).

Reactive oxygen species (ROS) cause cellular aging, mutagenesis, carcinogenesis, and coronary heart disease. This is likely due to destabilization of membranes, DNA and protein damage, and low-density lipoprotein (LDL) oxidation (**Heim *et al*., 2002**).

HHcy produces a rise in the creation of H2O2 and a reduction in the activity of the main antioxidant enzymes, glutathione peroxidase, superoxide dismutase, and catalase, thus promoting the generation of oxidative stress (**Rodrigo *et al*., 2003**).

Many studies have demonstrated that several phenolic antioxidant and plant extracts like coffee, catechin, and chlorogenic acid reduce plasma Hcy levels (**Nygard *et al*., 1997; Noll *et al*., 2011; Kim *et al*., 2012; Noll *et al*., 2013**), plants were an essential source of new

pharmacologically active compounds until recently, therefore many blockbuster medications are directly or indirectly derived from plants (**Veeresham, 2012**).

Astragalus armatus Willd (Fabaceae), popularly known as "ketad," is endemic species in Algeria. In addition, the aerial parts of *A. armatus* contain number of bioactive compounds including flavonol glycosides and triglycoside such as narcissin, nikotiflorin, and mauritianin. A new acylated flavonol triglycoside, was isolated from the aerial parts of *A. armatus* as well as ten known compounds, one phenolic compound, one flavonol-aglycone and eight flavonol-glycosides (**Khalfallah *et al*., 2014**). The flavonoid isorhamnetin was obtained from ethyl acetate extract of *A. armatus* which indicated the highest antioxidant activity in DPPH, ABTS and CUPRAC assays, and the n- butanol fractions led to the isolation of six compounds (four flavonoids including two triglycosyles, a saponin and a cyclitol) (**Labed *et al*., 2016**).

The main objectives of this thesis are:

- ✓ Induce hyperhomocysteinemia by administration of high dose of L-methionine (400 mg/kg), in an *in vivo* animal;
- ✓ Examine the effect a high dose of L- methionine (400 mg/kg) on some biochemical parameters such as plasma Hcy, triglycerides (TG), Total cholesterol (Tch), low density lipoprotein (LDL-c), high density lipoprotein (HDL-c), reduced glutathione (GSH), and catalase (CAT) in vivo;
- ✓ Examine the effect of a high dose of L-methionine (400 mg/kg) on different sections of aorta, heart and liver;
- ✓ Evaluate the protective effect of ethyl acetate (EtOAc) extract of *A. armatus* plant (100 mg/kg) on the plasma Hcy, lipid status, antioxidant status (GSH and CAT) on hyperhomocysteinemia caused by the high dose of L-methionine (400 mg/kg);
- ✓ Evaluate the protective effect of (EtOAc) extract of *A. armatus* (100 mg/kg) on histological abnormalities of aorta, heart and liver caused by the high dose of L-methionine (400 mg/kg);
- ✓ Evaluation of antioxidant activity of n-butanol (n-BuOH) extract of *A. armatus* in *vitro*;
- ✓ Evaluation of antiproliferative activity of (n-BuOH) extract of *A. armatus* on HeLa cells (human cervix carcinoma) and C6 cells (rat brain tumor).

CHAPTER I

Literature

Review

1. Homocysteine

1.1 Definition of homocysteine

Homocysteine (Hcy) is a sulfur amino acid discovered in 1933 by Du Vigneaud and which is not involved in protein synthesis (**Pellanda, 2012**), obtained from metabolism of essential aminoacid and obtained via diet in our body (**Kathpalia *et al.*, 2022**), structurally, it closely resembles methionine and cysteine; all three amino acids contain Sulfur (**Çelik *et al.*, 2017**).

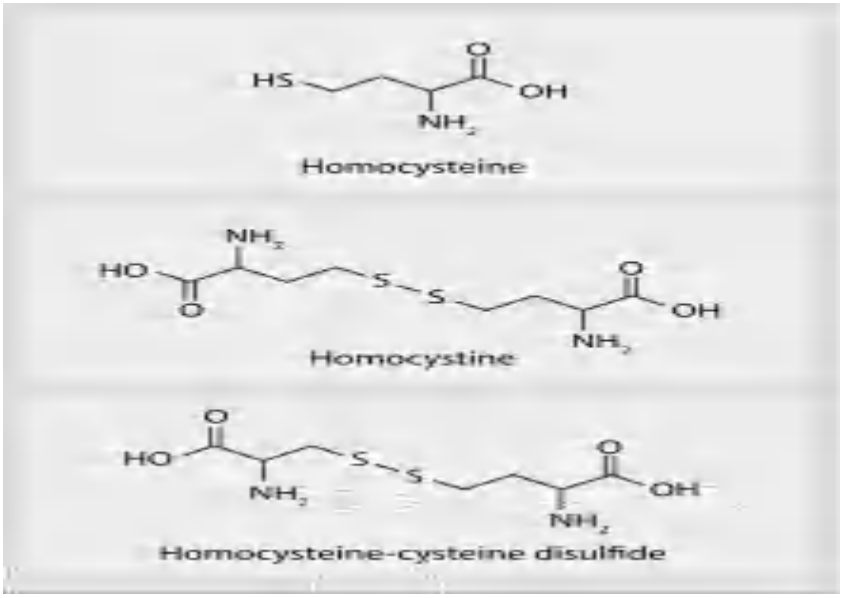

Figure 01. Forms of homocysteine in the blood (**Kathpalia *et al.*, 2022**).

Intracellular homocysteine is secreted at high concentrations and metabolized by one of the two pathways: remethylation or transsulfuration (Figure 02) (**Kathpalia *et al.*, 2022**).

Hcy is the principal factor related or implicated in a several metabolic pathologies such as atherosclerosis, thrombosis, diabetes, alzheimer, cerebral, and cardiovascular diseases (**Donald, 1998; Van Dam *et al.*, 2009; Buysschaert *et al.*, 2007**).

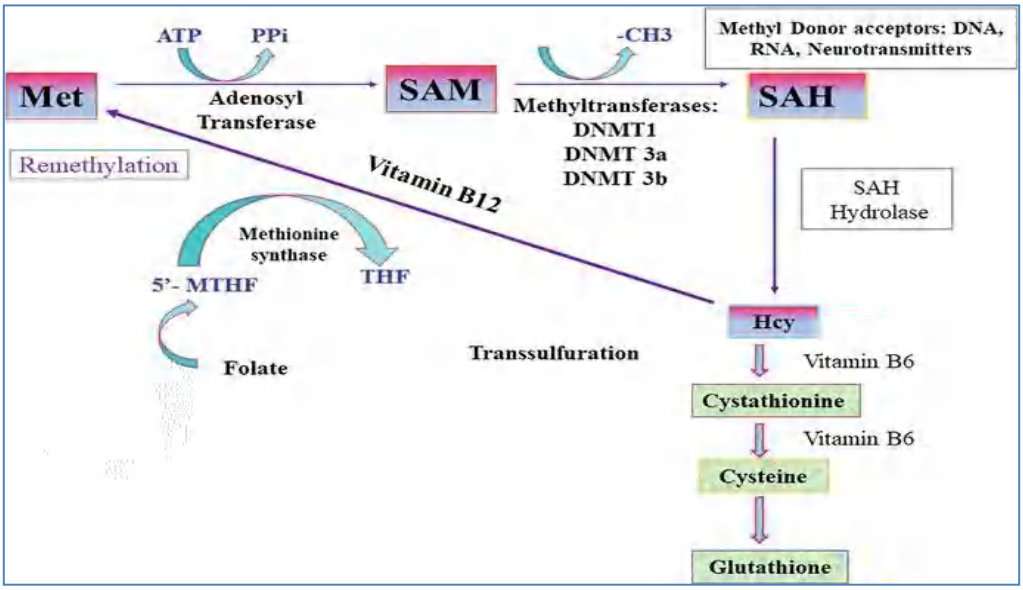

Figure 02. Homocysteine mechanism of breakdown (**Hasan *et al*., 2019**).

1.2. Structure and forms of homocysteine

Homocysteine is the term for both the sulfhydryl or reduced form and the disulfide or oxidized form. Both cysteine and proteins containing reactive cysteine residues have disulfide forms (protein- bound homocysteine). The latter oxidized forms are referred to as mixed disulfides (Figure 02) (**Donald, 1998**). The plasma contains homocysteine in four different forms: free homocysteine (1%), homocysteine disulfide (10%), mixed homocysteine-cysteine disulfide (10%), and the protein-bound form (80%) (**Kang and Rosenson, 2018**) (Table. 01).

Table 01: Constituents of total plasma homocysteine and percentage of composition (**Donald, 1998**).

Reduced:		
Homocysteine	$^{-}OOCCH(NH_3^+)CH_2CH_2-SH$	1 %
Oxidized:		
Homocystine	$^{-}OOCCH(NH_3^+)CH_2CH_2-S-S-CH_2CH_2(NH_3^+)COO^{-}$	5-10 %
Mixed-disulfides:		
Protein-bound Homocysteine	$^{-}OOCCH(NH_3^+)CH_2CH_2-S-S-$Protein	80-90 %
Cysteine-Homocysteine	$^{-}OOCCH(NH_3^+)CH_2CH_2-S-S-CH_2CH(NH_3^+)COO^{-}$	5-10 %

1.3. Metabolic pathway of homocysteine

It is important to note that methionine is the only source of homocysteine, homocysteine is a point of junction of two pathways: the methionine cycle (remethylation pathway) and the transsulfuration sequence (transsulfuration pathway) (Figure 02) **(Selhub, 2008)**, remethylation to methionine, which needs folate and vitamin B12 (or betaine in an alternative reaction); and transsulfuration to cystathionine, who needs pyridoxal-5′-phosphate (**Selhub, 1999)**. This reaction mainly takes place in the liver and to a smaller extent in the kidney and probably in the brain **(Miller, 2013)**.

1.3.1. Remethylation pathway

Under conditions of low protein intake, homocysteine is metabolised firstly by one of two methionine protecting remethylation pathways **(Finkelstein, 1998)**. In remethylation, homocysteine acquires a methyl group from N-5-methyl-tetrahydrofolate (MTHF) or from betaine to form methionine. The reaction with MTHF occurs in all tissues and is vitamin B12-dependent, while the reaction with betaine is confined mainly to the liver and is vitamin B12-independent. A considerable proportion of methionine is then activated by ATP to form S-adenosylmethionine (SAM). SAM serves primarily as a universal methyldonor to a variety of acceptors including guanidinoacetate, nucleic acids, neurotransmitters, phospholipids, and hormones. S-adenosylhomocysteine (SAH), the by-product of these methylation reactions, is subsequently hydrolyzed, thus regenerating homocysteine, which then becomes available to start a new cycle of methyl-group transfer (Figure 03) **(Selhub, 2008)**.

1.3.2. Transsulfuration pathway

When the remethylation pathway is saturated, or when cysteine is required, homocysteine is converted to cystathionine (and then cysteine) by cystathionine β-synthase (CβS) **(Finkelstein, 1998)**. In the transsulfuration pathway, homocysteine condenses with serine to form cystathionine in an irreversible reaction catalyzed by the pyridoxal-5′-phosphate (PLP)-containing enzyme, cystathionine β-synthase (CβS). Cystathionine is hydrolyzed by a second PLPcontaining enzyme, gamma-cystathionase, to form cysteine and alpha-ketobutyrate. Excess cysteine is oxidized to taurine and inorganic sulfates or excreted in the urine. Thus, in addition to the synthesis of cysteine, this transsulfuration pathway effectively catabolizes excess homocysteine which is not required for methyltransfer, and delivers sulfate for the synthesis of heparin, heparan sulfate, dermatan sulfate, and chondroitin sulfate. It is important to note that since homocysteine is not a normal dietary constituent, the sole source of homocysteine is methionine (Figure 03) **(Selhub, 2008)**.

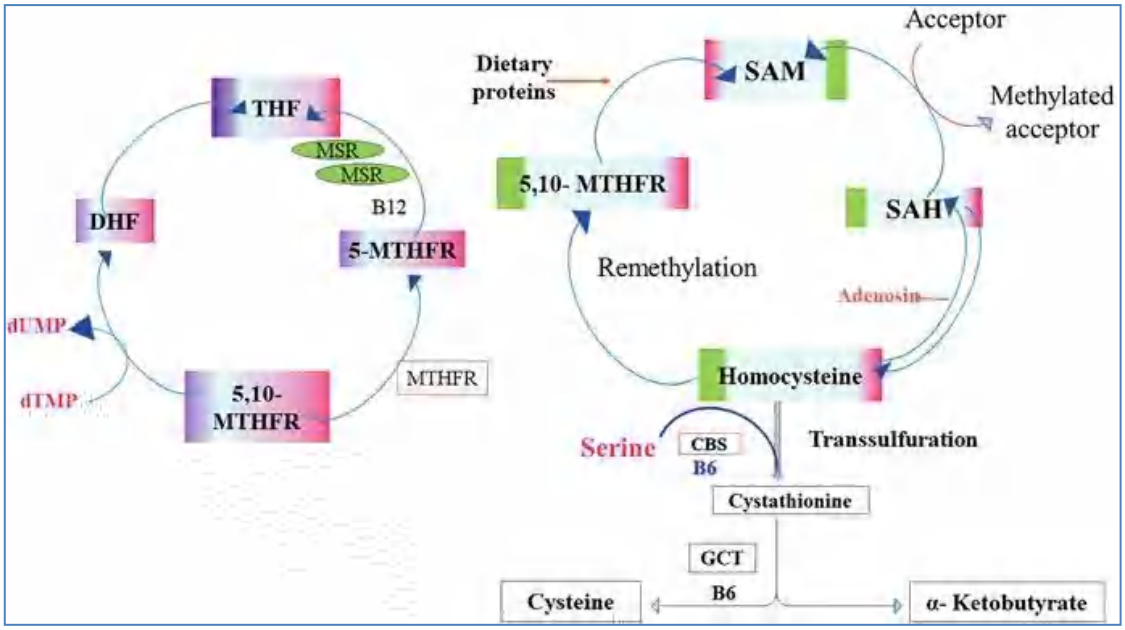

Figure 03. Metabolism of homocysteine (**Kathpalia *et al.*, 2022**).

1.4. Metabolic regulation

The distribution of substrate between competing reactions at two metabolic sites is the mechanism for the regulation of homocysteine (**Finkelstein, 1990**). The ultimate source of homocysteine is dietary methionine. In remethylation, homocysteine acquires a methyl group from N-5-methyltetrahydrofolate or from betaine to form methionine. The reaction with N-5-methyltetrahydrofolate occurs in all tissues and is vitamin B12 dependent, whereas the reaction with betaine is confined mainly to the liver and is vitamin B12 independent (**Selhub, 1999**). Methionine is first activated by the addition of an adenosyl group (from adenosine triphosphate) to form S-adenosylmethionine(SAM), Aproduct of all SAM-dependent methylation reactions is S-adenosylhomocysteine(SAH),which in turn is metabolized to form adenosine and homocysteine. (**Miller, 2013**).

In the transsulfuration reaction, methionine is converted to homocysteine which irreversibly condenses with serine to form cystathionine. This reaction is catalyzed by the B6-dependent enzyme cystathionine β-synthase (CBS). Cystathionine is hydrolyzed to cysteine by the enzyme y-cystathionase (**Roblin, 2007**). In the remethylation reaction, methionine is converted to homocysteine which irreversibly condenses with serine to form cystathionine. This reaction is catalyzed by the B6-dependent enzyme cystathionine β-synthase (CβS). Cystathionine is hydrolyzed to cysteine by the enzyme y-cystathionase (**Guba *et al.*, 1996**).

An addition al level of control on homocysteine metabolism is exerted by oxidative stress, which reduces methionine synthase activity. This may occur by oxidative in activation of the vitaminB12 cofactor or by the oxidation of cysteine residues that are important for zinc binding. By inhibiting methionine synthase, oxidative stress tends to divert homocysteine toward cystathionine synthesis away from methionine synthesis. This serves to increase the synthesis of glutathione, a product of homocysteine metabolism through the transsulfuration pathway and an important intracellular antioxidant (**Miller, 2013**).

2. Cardiovascular system

It is a closed circuit of vessels that permits the life of each cell in the human organism and in all mammals. The blood is kept in continuous motion from the left heart, via the aorta, arteries, arterioles, capillaries, venules, veins, vena cava, to the right heart, through the pulmonary artery to the lungs, and finally, through the pulmonary vein, back to the left heart (Figure 04) (**Stefanovska, 1999**).

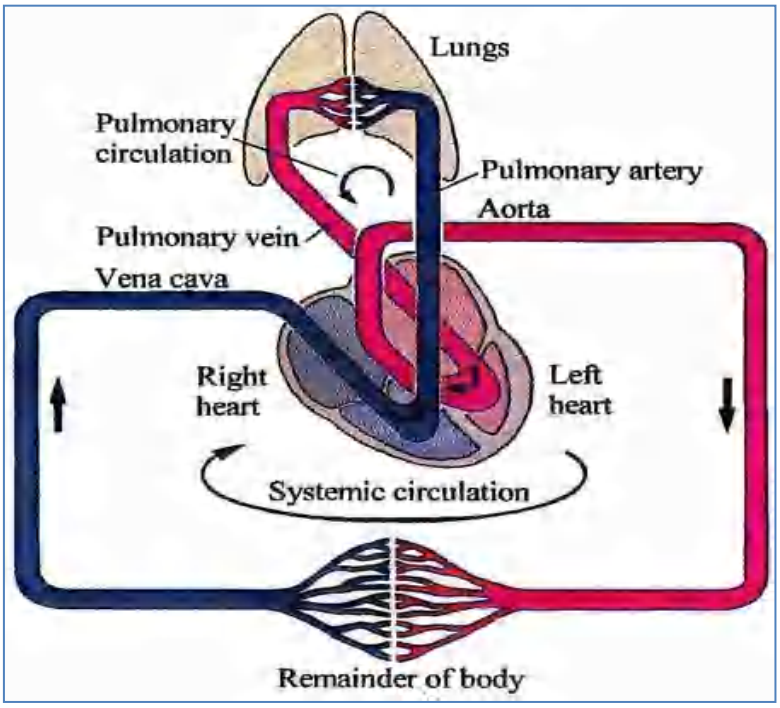

Figure 04. Blood circulates through the cardiovascular system (**Stefanovska, 1999**).

2.1. Heart

The heart is the pump that distributes blood throughout the body. In humans, all minutes, 5 liters of blood are pumped from the heart and passed through the blood vessels to irrigate all tissues and organs of the body (**Ghandour, 2013**); it receives deoxygenated blood from the body, sends it to the lung, receives oxygenated blood from the lungs, and then pumps the oxygenated blood throughout the body **(Arackal and Alsayouri, 2022**). The heart is situated in the chest, directly above the diaphragm, in the area of the thorax known as the mediastinum, especially the middle mediastinum (**Shah *et al.*, 2009**); the heart is separated into four distinct chambers, each with a varied thickness of muscle walls. Small chambers with thin walls, the left atrium (LA) and right atrium (RA), are situated directly above the left ventricle (LV) and right ventricle, respectively. Most of the work is performed by the ventricles, which are larger chambers with thick walls (Figure 05) (**Edwards, 1984**).

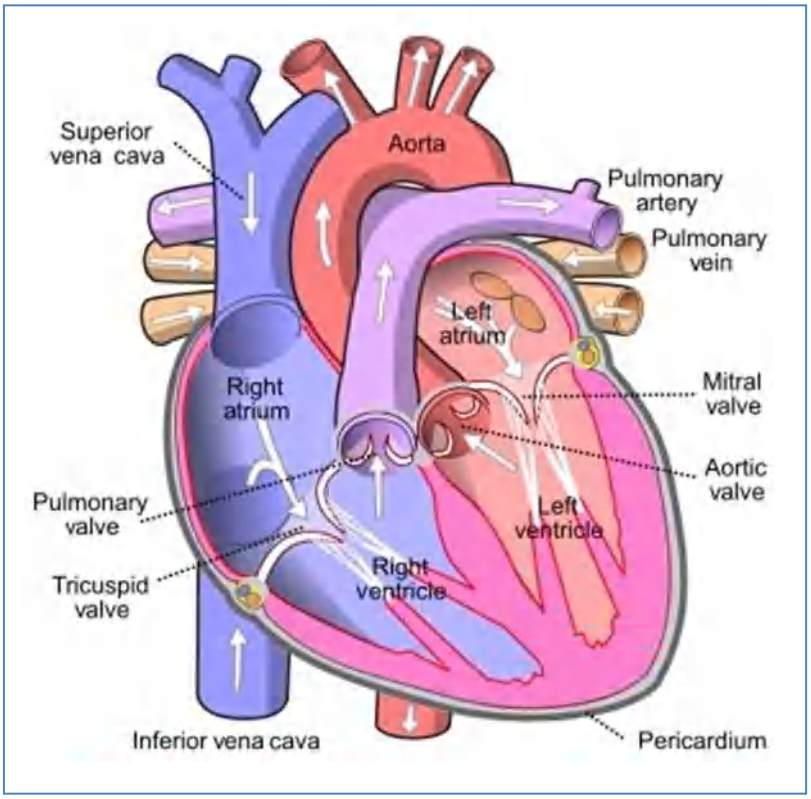

Figure 05. The human heart anatomy and blood flow (**Bamalan *et al.*, 2022**).

2.1.1. Histological structure of the heart

The base of the heart contains a highly dense structure known as the fibrous or cardiac skeleton. The fibrous skeleton has several functions, including providing a solid framework for cardiomyocytes,

anchoring the valve leaflets, and acting as electrical insulation separating the conduction in the atria and ventricles (**Saremi *et al*., 2017).**

The wall of the heart is composed of three layers, which are in order: The epicardium is the external lining of the heart chambers and is formed by the visceral layer of the serous pericardium. The intermediate layer of the heart, known as the myocardium, is composed of three distinct layers of muscle; the endocardium is the innermost layer of the heart and is composed of the endothelium and sub-endothelial connective tissue **(Shah *et al*., 2009**).

The wall of the heart separates into the following layers: epicardium, myocardium, and endocardium. These three layers of the heart are embryologically equivalent to the three layers of blood vessels: tunica adventitia, tunica media, and tunica intima, respectively, a double-layer, fluid-filled sac known as the pericardium surrounds the heart (**Arackal and Alsayouri, 2022**); the epicardium comprises the visceral pericardium, underlying fibro-elastic connective tissue, and adipose tissue (**Rodriguez and Tan, 2017**). The myocardium is the thickest wall of the heart. It is structured in the form of myocardial trabecular made up cardiac muscle cells anastomosed between these spans (**Schaffler *et al*., 2004**). The endocardium is constituted of the endothelium and a layer of subendothelial connective tissue, the subendocardium is located between the endocardium and the myocardium and contains the impulse-conducting system (Figure 06) (**Arackal and Alsayouri, 2022**).

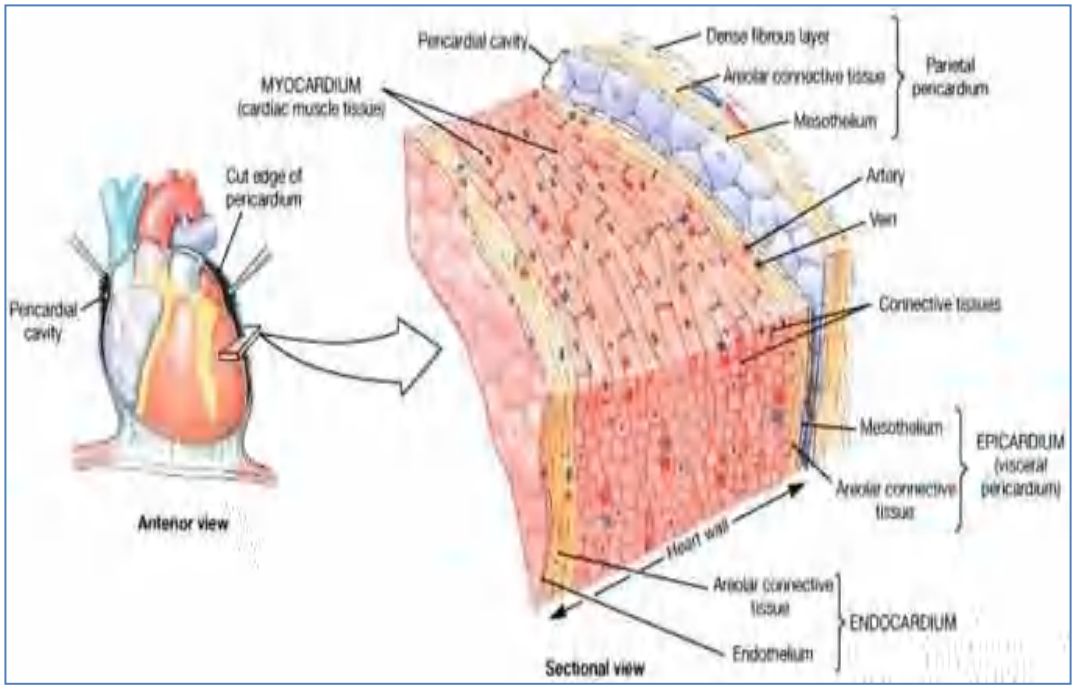

Figure 06. Histological structure of the heart (**Net 1**).

2.2. Blood vessels

The peripheral vascular system (PVS) consists of all blood vessels found outside of the heart. The peripheral vascular system is divided into several categories: The aorta and its branches (arteries), the arterioles, the capillaries, the venules and veins (Figure 08) (**Tucker *et al.*, 2021**).

2.2.1. Arteries

There are two main types of arteries found in the body: the elastic arteries, and the muscular arteries. Muscular arteries contain more smooth muscle cells in the tunica media layer than the elastic arteries. Elastic arteries are those nearest the heart (aorta and pulmonary arteries) that contain much more elastic tissue in the tunica media than muscular arteries (**Tucker *et al.*, 2021**). Arteries are formed of an internal endothelium (tunica intima) covered by internal elastic tissue, a smooth muscle cell layer (tunica media), external elastic tissue, and fibrous connective tissue (tunica adventitia) (**Cleaver and Krieg, 1999**).

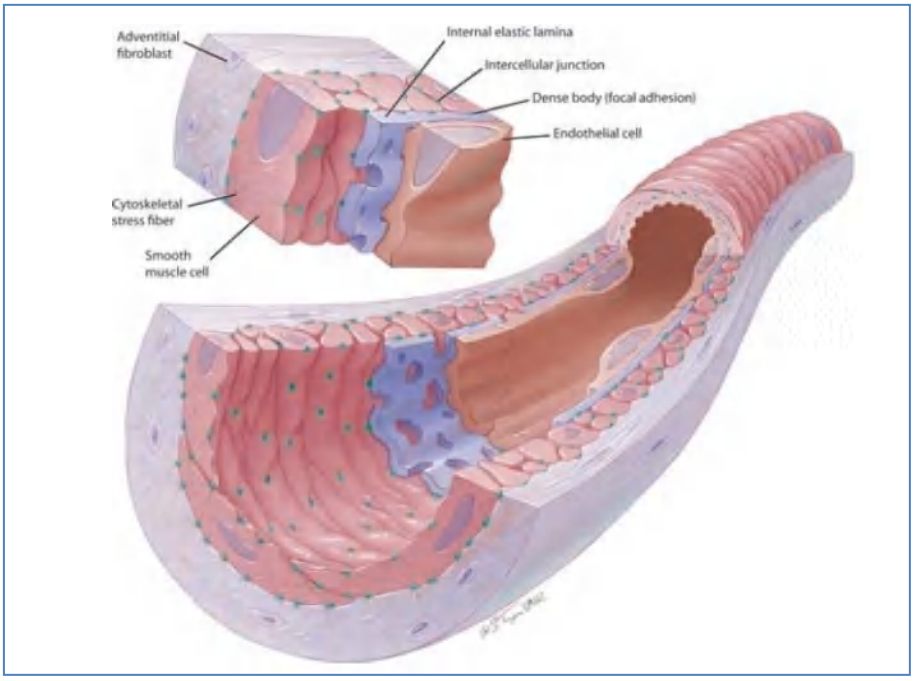

Figure 07. Anatomy of resistance arteries (**Martinez-Lemus, 2012**).

2.2.2. Arterioles

Arterioles are the blood vessels in the arterial side of the vascular tree that are located proximal to the capillaries and, in conjunction with the terminal arteries, provide the majority of resistance to blood flow (**Martinez- Lemus, 2012**). Arterioles are important vital hemodynamic regulators because they contribute to the upstream pressure and the regional distribution of blood and provide around 80%

of the total resistance to blood flow through the body (**Christensen and Mulvany, 2001; Meininger *et al*., 1984**).

2.2.3. Capillaries

Capillary walls are only one endothelial cell in thickness and have no elastic, muscle, or fibrous tissue (**Gavaghan, 1998**), the exchange of nutrients and metabolites starts primarily via diffusion because of the thin walls of the capillary. Blood flow via capillaries is regulated by the arteriolar lumen (**Tucker *et al*., 2021**). Each capillary has an arterial end and a venous end. At the arterial end, oxygen and nutrients are forced into the tissues, and the venules at the venous end collect cellular wastes and carbon dioxide (CO) (**Gavaghan, 1998**).

2.2.4. Venules

Venules, the smallest veins, receive blood from the capillaries and convey it to larger vessels that increase in size as they travel from the tissues to the heart (**Gavaghan, 1998**). Additionally, they play a role in the exchange of oxygen and nutrients for water products. Between capillaries and venules, there are post-capillary sphincters; the venule is very thin-walled and easily prone to rupture with excessive volume (**Tucker *et al*., 2021**).

2.2.5. Veins

The venous wall is thinner than the arterial wall and has a lower tone than the arterial wall **(Maggisano and Harrisson, 2004)**, this feature permits the veins to hold an extremely high percentage of the blood in circulation. The venous system can accommodate a high blood volume at low pressures, known as high capacitance (**Tucker *et al*., 2021**). Veins contain three layers as well (tunica intima, tunica media and tunica adventitia); however, there is only a thin layer of smooth muscle and few elastic fibers in the tunica media of a vein. Veins have valves consisting of pocket-like flaps that help blood flow return to the heart and stop backflow (Figure 08) (**Gavaghan, 1998**).

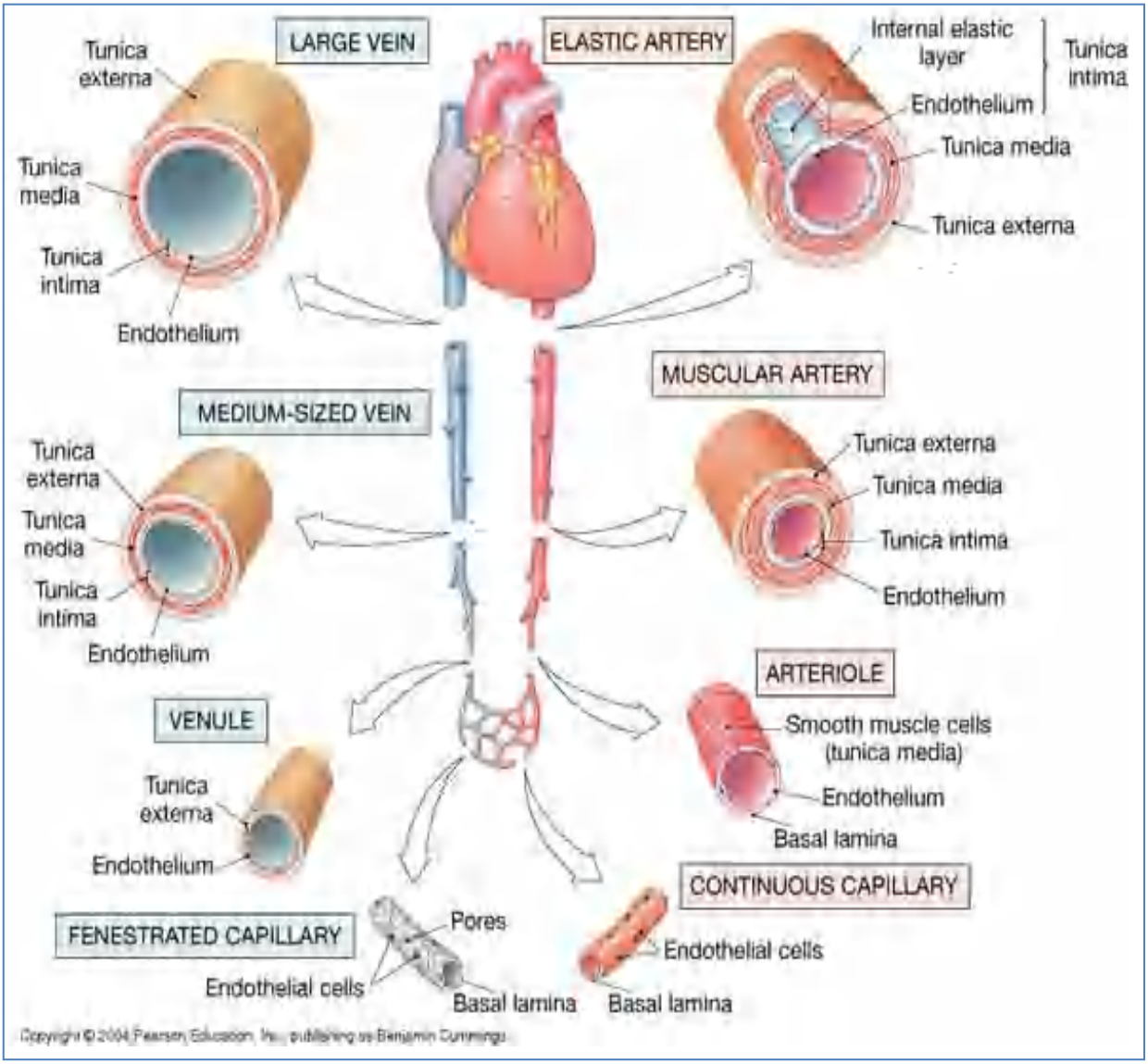

Figure 08. The different types of blood vessels and their layers (**Net 2**).

2.3. Histology of blood vessels

The artery and vein walls are composed of three layers: the tunica externa (tunica adventitia), the tunica media is the intermediate layer of the vessel wall and the tunica intima is the innermost layer of the vessel wall (Figure 08) (**Taylor *et al.*, 2022**).

2. 3. 1. Histological structure of the aorta

The aorta is one of the elastic/conductive arteries which have the largest diameter and the greatest elasticity; they are large caliber vessels, with a round lumen, and whose wall is relatively thin. We classically distinguish three concentric layers of cells, from the lumen to the periphery: the intima, the media and the adventitia (**Hill *et al.*, 1989**).

2.3.1.1. Intima

The intima is the internal tunic, composed mainly of collagen and a continuous monolayer of endothelial cells, it rests on a thin layer of connective tissue called the basal lamina. The latter is it supported by an elastic lamina called the internal elastic lamina, acting as a boundary between the endothelium and the extracellular matrix (ECM) of the media (**Hill *et al.*, 1989; Dadoune *et al.*, 1990**).

2.3.1.2. Media

The media is the middle tunic, and the thickest of the arterial wall, made up of smooth muscle cells (SMC) and an ECM rich in elastin and collagen fibers, it is delimited by two elastic borders; an internal elastic blade on the side of the vascular lumen and an external elastic blade. This layer is considered the active part of the artery (elasticity, modification of caliber, etc.) (**Hill *et al.*, 1989; Dadoune *et al.*, 1990**).

2.3.1.3. Adventitia

The adventitia is the outer tunic. It is made up of loosely organized connective tissue, rich in collagen and elastic fibers, and containing fibroblasts, adipocytes, immune cells, nerve endings and vasa vasorum (feeding vessels). It ensures the anchoring of the arteries to the surrounding structures (**Hill *et al.*, 1989; Dadoune *et al.*, 1990**).

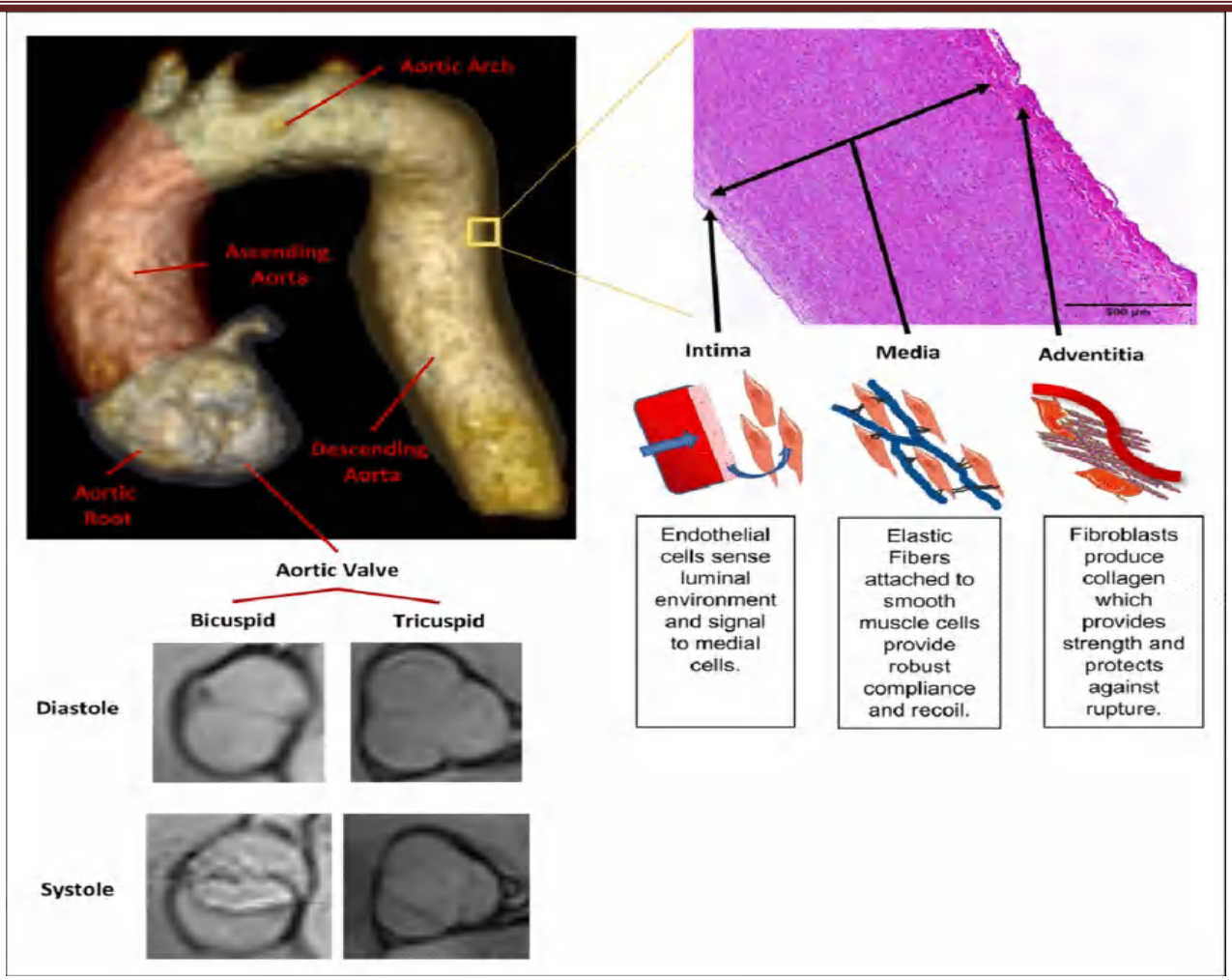

Figure 9. Anatomy and histology of the thoracic aorta (**Fletcher *et al.*, 2020**).

3. Hyperhomocysteinemia

3.1. Definition

Hyperhomocysteinemia, defined as an elevated concentration of homocysteine inthe fasting state or after methionine loading (**Van Der Griend *et al.*, 1998**). Between fasting individuals, "Normal" tHcy levels are typically between 5 and 15 µmol/L, and higher fasting levels are arbitrarily classed as moderate (16-30), intermediate (31-100), and severe (>100 µmol/L) hyperhomocysteinaemia (**Hankey and Eikelboom, 1999**).

The appearance of Hyperhomocysteinemia indicates that homocysteine metabolism has been disrupted in some way and that the export mechanism is disposing of excess homocysteine in the blood (**Selhub, 2008**).

3.2. Factors influencing plasma total homocysteine level

3.2.1. Inherited

Hyperhomocysteinemia is caused by inherited deficiencies of enzymes in the methionine homocysteine pathway, the two most frequently affected being (CβS) and 5, 10 methylenetetrahydrofolate reductase (MTHFR) (**Haynes, 2002**).

Severe increases in plasma homocysteine are detected in individuals with homozygous genetic defects affecting cystathionine β-synthase (CβS), the gene for this enzyme is inherited in an autosomal recessive fashion and demonstrates observed genetic heterogeneity (**Rees,1993**), hyperhomocysteinemia can also be caused by deficiencies of enzymes in the remethylation pathway (**Rees and Rodgers, 1993**). MTHFR or any of several enzymes are responsible for converting vitamin B12 into its methionine synthase-associated cofactor form, these autosomal recessive genetic disorders (**Miller, 2013**), homozygous deficiency of this enzyme occurs in the general population at a rate of around one-tenth that of (CβS) deficiency (**Skovby, 1989**).

3.2.2. Acquired

Hyperhomocysteinemia is caused by dietary insufficiency or malabsorption of folate, vitamin B12, or vitamin B6 (**Pancharuniti *et al*., 1994; Rimm *et al*., 1998**). Vitamin B6 deficiency may result in reduced activity of CβS (**Rimm *et al*., 1998**).

Certain medications can interfere with normal Hcy metabolism, several of these medication effects are due to secondary functional vitamin deficiencies (**Stipanuk, 2004**). Old age, male sex, smoking, high blood pressure, elevated cholesterol, and lack of exercise are all associated with elevated tHcy levels **(Nygard *et al*., 1995**); hyperhomocysteinemia has also been related to zinc insufficiency, leukemia, psoriasis, and antifolate medications (Table. 02) (**Ueland and Refsum, 1989**).

Table 02: Causes of elevated plasma homocysteine levels (**Keebler *et al*., 2001**).

Nutritional deficiencies	**Genetic**
Folate	Trans-sulfuration abnormalities: cystathionine β-synthase deficiency or mutations
Vitamin B_{12}	Remethylation disorders (eg, defective vitamin B_{12} transport or coenzyme synthase, defective methionine synthase)
Vitamin B_6	Mutation in *MTHFR* gene
Medications	Cobalamin mutations
Methotrexate	
Phenytoin and carbamazepine	**Demographic characteristics**
Nitrous oxide	Age
Theophylline	Male sex
Metformin	Tobacco use
Cholestyramine (colestipol, colesipol and niacin	Physical inactivity
	Postmenopausal status
Disease states	
Chronic renal failure	**Acute-phase response to illness**
Acute lymphoblastic leukemia	
Malignancies	
Hypothyroidism	**Increased methionine consumption**
Type 2 diabetes	
Type 1 diabetes and nephropathy	
Systemic lupus erythematosus	
Severe psoriasis	
Transplantation	

3.3. Therapy of hyperhomocysteinemia

The combination of the three vitamins (folie acid, vitamin B-12 and vitamin B-6) reduced circulating homocysteine concentrations by 49.8% (**Ubbink *et al*., 1994**), one strategy for lowering homocysteine levels is to facilitate homocysteine catabolism to cysteine through the transsulfuration pathway (**Rees and Rodgers, 1993**).

Analytical techniques for treating hyperhomocysteinemia are reviewed in which stepwise administration with 5-methyltetrahydrofolate (5- MTHF) dietary dosages, and based on clinical and laboratory evaluations, betaine is provided singly or in combination (**Kang and Rosenson, 2018**). Penicillamine treatment may be beneficial for Non-responsive patients; penicillamine decreases homocysteine levels by forming a mixed disulfide with the free thiol (homocysteine penicillamine MDS) which may increase renal excretion (**Ueland and Refsum, 1989; Kang *et al*., 1982**).

4. Pathologies associated with homocysteine

4. 1. Hyperhomocysteinemia and cardiovascular diseases

Cardiovascular disease (CVD) is the main cause of death in the United States and most Western countries (**Donald, 1998**). In 1964, the association of homozygous homocysteinemia to vascular

disease and thrombosis was first described pathologically (**Gibson *et al.*, 1964**), the risk of cardiovascular disease has been associated with higher blood Hcy levels (**Peng *et al.*, 2015**). Atherosclerosis is the main predominant pathology of cardiovascular disease (**Gerdes, 2005**), hyperhomocysteinemia is a risk factor for atherosclerotic vascular disease, and it is associated with endothelial dysfunction (**Sydow *et al.*, 2003**).

4.1.1. Atherosclerosis

Arteriosclerosis is described as chronic inflammatory damage to the arterial intima with elevated plasma permeability (**Schaffer *et al.*, 2014)**, it is characterized by a thickening of the arterial wall due to smooth muscle cell proliferation, lipid deposits, and fibrosis (**Davies, 1996**). The hallmark of atherosclerosis is the atherosclerotic plaque, which contains lipids (intracellular and extra cel-lular cholesterol and phospholipids), inflammatory cells (e.g., macrophages, T-cells), smooth muscle cells, con-nective tissue (e.g., collagen, glycosaminoglycans, elastic fibers), thrombi, and calcium deposits. Homocysteine is known as an independent risk factor for atherosclerosis (**Tayal *et al.*, 2011**). The correlation between hyperhomocysteinemia and atherosclerotic disease was first proposed more than 40 years ago. It was first identified by McCully in 1969 (**Pang *et al.*, 2014**).

Arteriosclerotic plaques were found in the aorta and arteries of rabbits givenhomocysteine thiolactone, methionine or homocysteic acid, both parenterally and in a synthetic diet (**Ganguly, 2015**). Baboons receiving homocysteine intravenously produce endothelial desquamation, increased consumption of platelets, and typical arteriosclerotic plaques are composed of diffuse fibrous plaques containing hyperplastic smooth muscle cells, elastic fibers, collagen, and glycosaminoglycans (**McCully and Wilsson, 1975**). Choline deficiency produces hyperhomocysteinemia because of reduced remethylation of homocysteine to methionine, which explains the origin of arteriosclerotic plaques in these animals (**Harker *et al.*, 1976**).

In vascular endothelial cells, homocysteine inhibits DNA synthesis and stops cell development in the G1 phase of the cell cycle (**Rinehart and Greenberg, 1949**). Additionally, homocysteine stimulates the MAP kinase signal transduction pathway and the induction of C-fos and C-my genes, which causes mitogenesis in vascular smooth muscle cells (**Starkebaum and Harlan, 1993**). Increased homocysteine level may cause hyperlipidemia by competing with PPAR ligands, like fibrates which are known to catabolise VLDL and triglycerides (**Upchurch *et al.*, 1997**). Homocysteine promotes the binding of lipoprotein to fibrin and the growth of smooth muscle cells, and tHcy levels correlate with levels of fibrinogen, an independent risk factor for atherosclerotic vascular disease (**James *et al.*, 1998**).

In addition, certain byproducts of homocysteine metabolism, such as homocysteine thiolactone, react with LDLto form aggregates that are taken up by macrophages. These macrophages are incorporated into foam cells in early atherosclerotic plaques. After incorporation, homocysteine thiolactone acylates proteins and enhances oxidation in the vessel wall, promotes DNA synthesis and proliferation of vascular smooth muscle cells, and inhibits DNA synthesis in endothelial cells, thus further accelerating the development of atherosclerotic plaques (**Temple *et al*., 2000**).

Homocysteine has been found to induce the expression of macrophage lipoprotein lipase (LPL) both at the transcription and translation level presumably via PKC activation. LPL is the major lipolytic enzyme involved in hydrolysis of triglycerides in lipoproteins. It is secreted by macrophages in atherosclerotic lesions and macrophage LPL produced in the vascular wall acts as a pro-atherogenic protein (**Sharma *et al*., 2006**). Hyperhomocysteinemia-related atherosclerosis mostly results from endothelial cell damage, platelet pathologic alterations have also been implicated in the disease (Figure 10) (**Temple *et al*., 2000**).

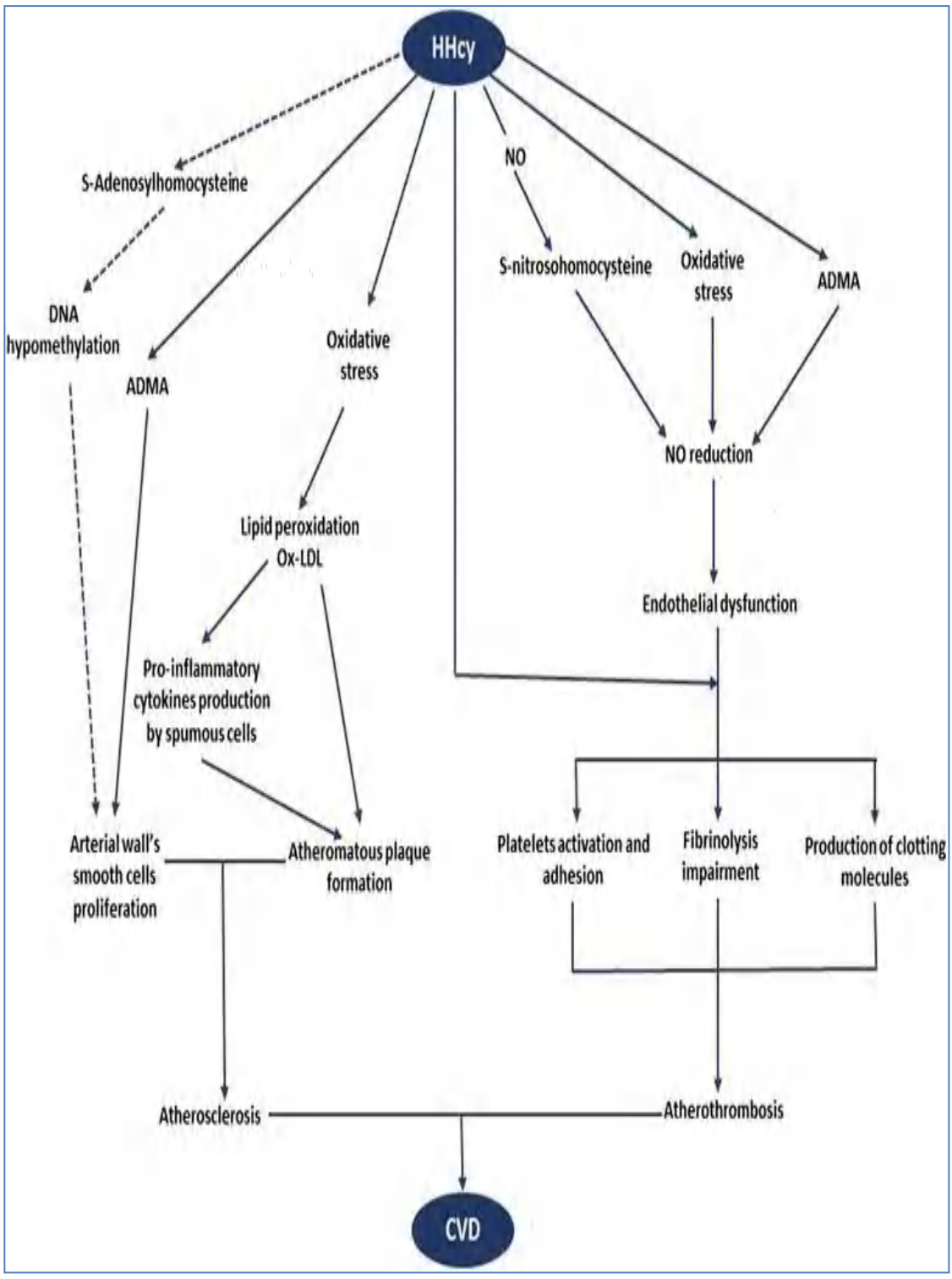

Figure 10. The causes of hyperhomocysteinemia (HHcy) and the mechanism of homocysteine (Hcy)–mediated endothelial cells (ECs) injury and its consequences for atherosclerosis (**Essouma and Noubian, 2015**).

4. 1. 2. Endothelial dysfunction

Endothelial dysfunction can be described as an imbalance between vasodilator and vasoconstrictor produced by the endothelium, and it has been regarded as the core systemic pathological status in the process of atherosclerosis and CVD (**Lai and Kan, 2015**).

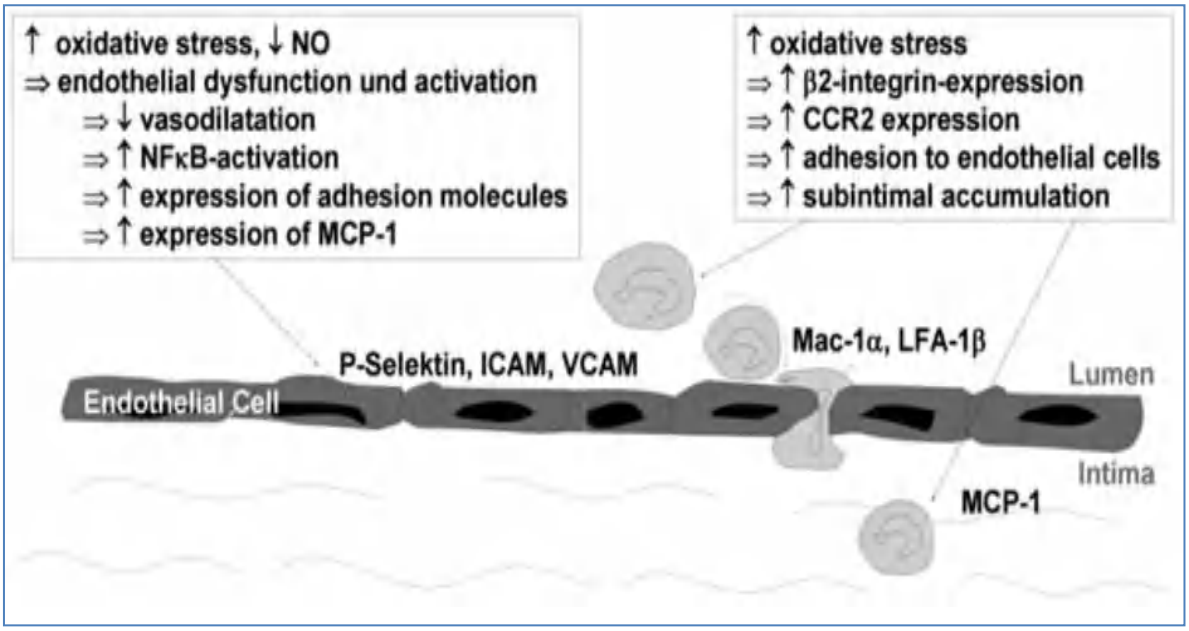

Figure 11. Effect of homocysteine on endothelial activation and the adhesion and transmigration of leukocytes to and through the vascular endothelium (**Papatheodorou, 2007**).

When excess homocysteine is present and homocysteine metabolism becomes deregulated, a major byproduct is homocysteine thiolactone, which reacts with lowdensity lipoprotein (LDL) to form LDL-homocysteine thiolactone aggregates. These are taken up by macrophages and subsequently incorporated into foam cells in early atherosclerotic plaques (**Welch *et al*., 1997**).

Hyperhomocysteinemia is related with endothelial dysfunction, mechanisms responsible for endothelial dysfunction in hyperhomocysteinemia may be caused by decreased bioavailability of NO, perhaps secondary to accumulation of the endogenous NO synthase inhibitor asymmetric dimethylarginine (ADMA) and augmented oxidative stress (**Sydow *et al*., 2003**).

The potential mechanisms by which elevated plasma homocysteine level leads to reduction in nitric oxide bioavailability include the disruptive uncoupling of nitric oxide synthase activity and quenching of nitric oxide by oxidative stress, the enzymatic inhibition by asymmetric dimethylarginine, endoplasmic reticulum stress with eventual endothelial cell apoptosis, and chronic inflammation/prothrombotic conditions (Figure 12) **(Lai and Kan, 2015)**.

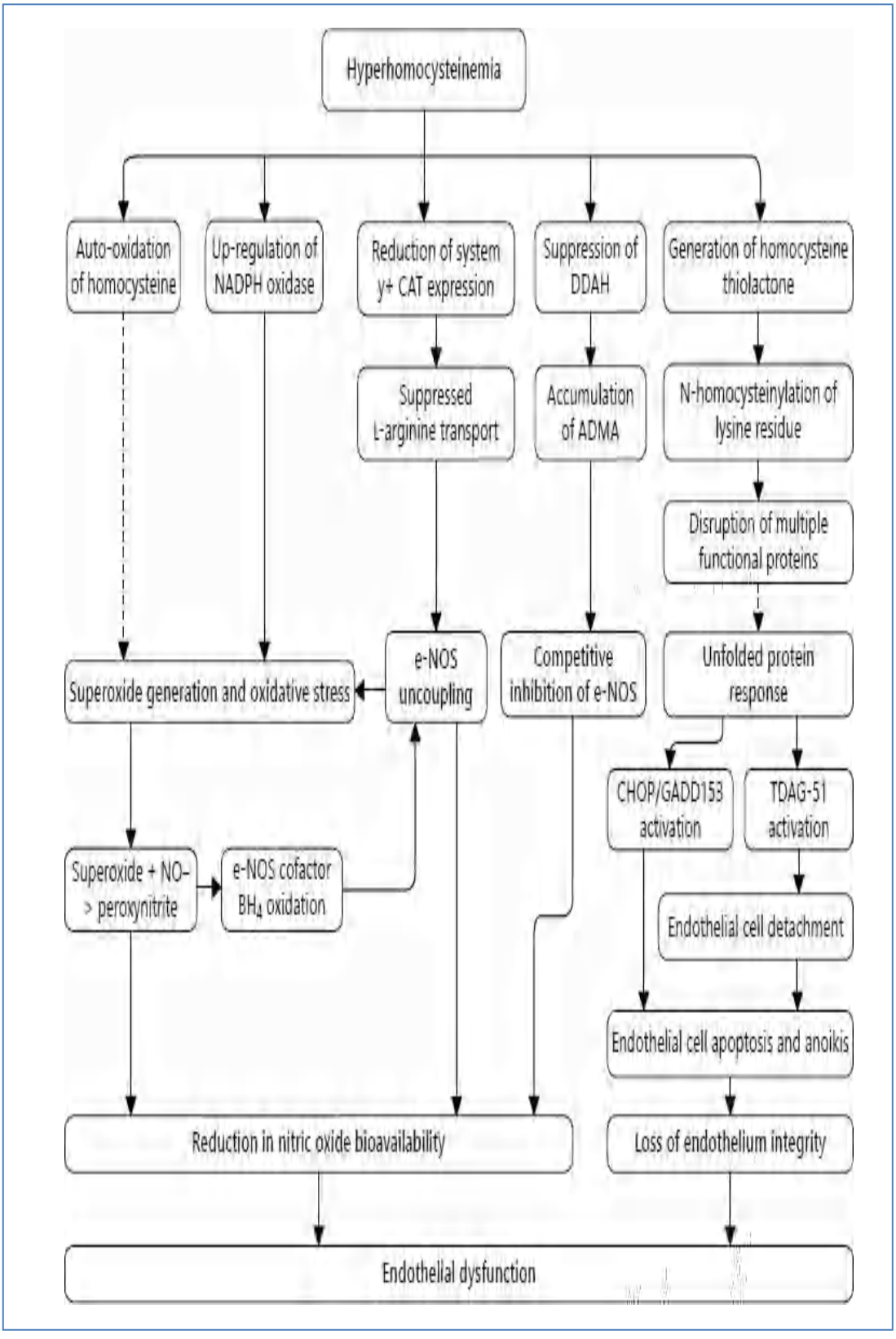

Figure 12. Mechanisms of homocysteine-induced endothelial dysfunction **((Lai and Kan, 2015).**

4.2. Hyperhomocysteinemia and hepatic disease

Liver plays an essential role in homocysteine metabolism (**Liu *et al.*, 2010**). Hyperhomocysteinemia causes a variety of clinical manifestations, notably liver damage; homocysteine is thought to be pathogenic because it can cause oxidative stress (**Noll *et al.*, 2011**). In the liver of rats with hyperhomocysteinemia, lipid peroxidation increased while total thiol content and antioxidant defenses were reduced.

Histological examination of liver tissue sections from hyperhomocysteinemic rats indicated the presence of an inflammatory infiltrate, fibrosis, and decreased content of glycogen/glycoprotein (**Matte´ *et al.*, 2009**). We have observed a marked reduction in the expression of the main genes involved in homocysteine metabolism in liver cirrhosis. In addition, Hcy-induced 1α (I) procollagen expression in the hepatic stellate cell (**García-Tevijano *et al.*, 2001**). After receiving a diet rich in methionine, inflammatory reactions, microvesicular steatosis, and hepatocyte degradation were observed in the liver (**Yalçınkaya *et al.*, 2009**). While hyperhomocysteinemia promotes mitochondrial oxidative stress and pro-apoptotic signals in the liver of CβS-deficient mice, a murine model of hyperhomocysteinemia, protective mechanisms may counteract these pro-apoptotic signals, causing chronic inflammation (**Hamelet *et al.*, 2009**).

Hyperhomocysteinemia decreased antioxidant defenses and total thiol content, and increased lipid peroxidation in liver of rats. Histological analysis indicated the presence of inflammatory infiltrate, fibrosis and reduced content of glycogen/glycoprotein in liver tissue sections from hyperhomocysteinemic rats **(Matte´ *et al.*, 2009**).

In the case of hyperhomocysteinemia (HHcy), down-regulated miR-212-5p particularly upregulates PSMD10 expression in hepatocytes. PSMD10 then activates ER stress through interacting with GRP78 and facilitates apoptosis of hepatocytes, by which Hcy induces liver damage (Figure 13) (**Xiao *et al.*, 2021**).

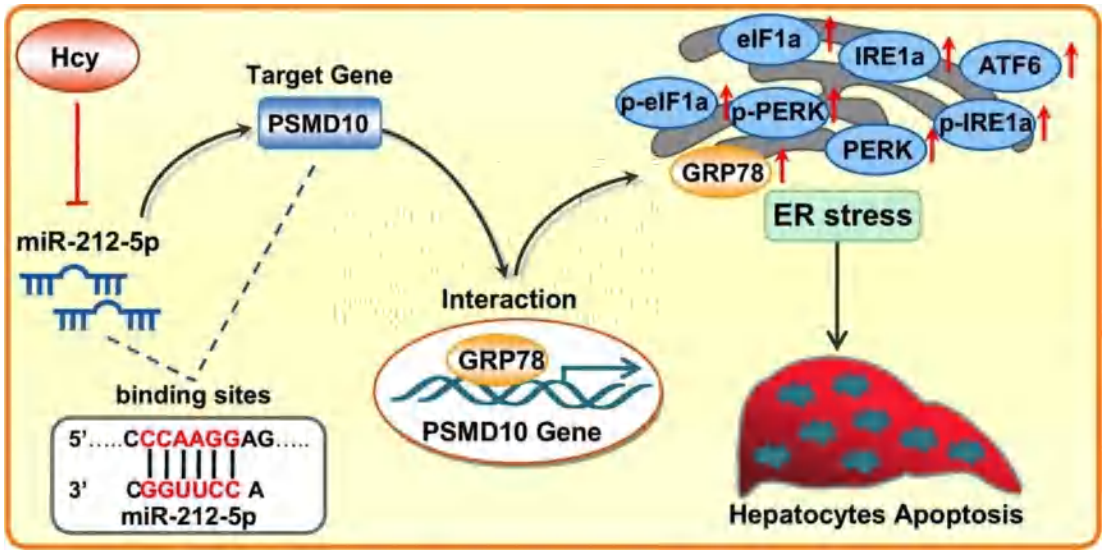

Figure 13. Interaction between PSMD10 and GRP78 accelerates endoplasmic reticulum stress-mediated hepatic apoptosis induced by homocysteine (**Xiao *et al.*, 2021**).

4.3. Hyperhomocysteinemia and carcinogenesis

Homocysteine is considered to be an important risk factor for cancer as well as cardiovascular diseases (**Oikawa *et al.*, 2003**). Different biochemical changes, including folate deficiency, oxidative stress, aberrant DNA methylation, and the formation of homocysteine thiolactone, have been identified in association with hyperhomocysteinemia, explaining why increased homocysteine eventually led to carcinogenesis (**Wu, 2002**), Several research has found that hyperhomocysteinemia influence on carcinogenesis is related to low folate levels and other vitamin B deficiencies produced by the exact metabolic mechanisms that cause hyperhomocysteinemia (Figure 14) (**Kathpalia *et al.*, 2022**), 5- MTHF reduction causes global genomic hypomethylation, which is an early and consistent event in carcinogenesis (Table 03**)**, the activation of proinflammatory genes due to region-specific hypomethylation is another mechanism that homocysteine might predispose to cancer (**Keshteli *et al.*, 2015**), for instance, it has been shown that MTHFR polymorphisms influence the chance that cancers would spread, with the association being that MTHFR isoforms that increase homocysteine levels typically increase the risk of cancer. Many research studied the epigenetic alterations linked to the progression of cancer and located the next Hcy level and an unchanged plasma level of Cys in cancer patients (**Kathpalia *et al.*, 2022**).

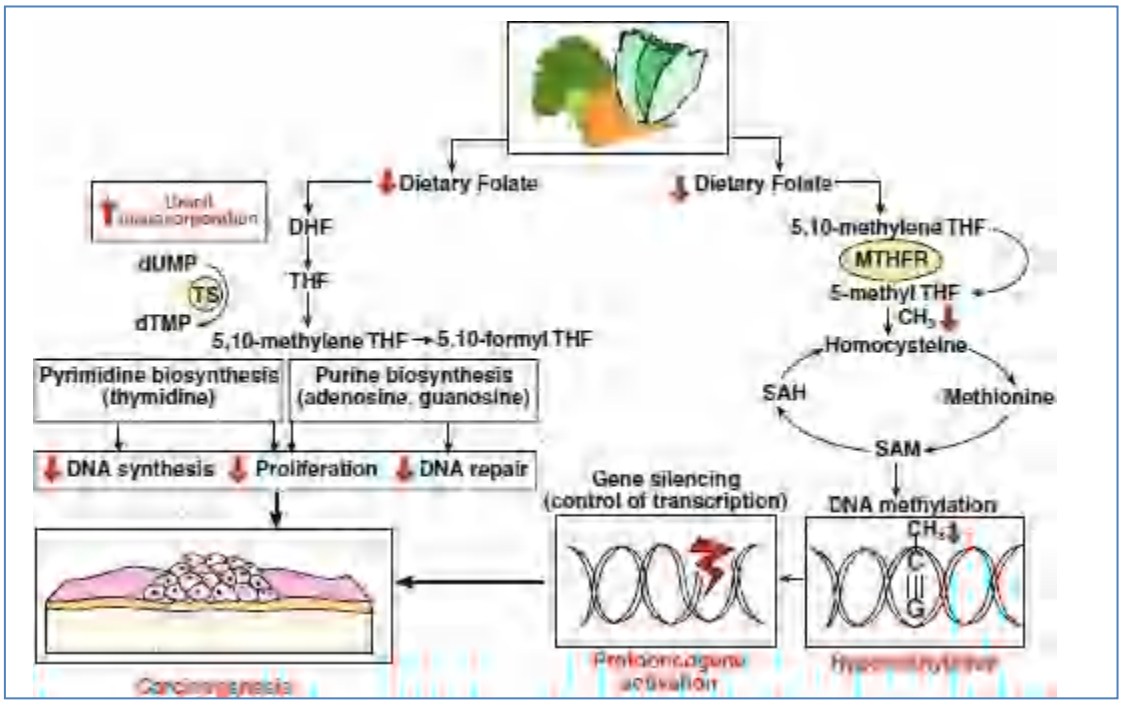

Figure 14. Low folate levels are associated with the impact of hyperhomocysteinemia on cancer development (**Duthie *et al.*, 2011**).

Table 03: Gene responsible for causing the disease (**Kathpalia *et al.*, 2022**).

Gene name	Type of polymorphism	Cancer type
Methylenetetrahydrofolate reductase (MTHFR)	677C → T 1298A → C 1793G → A	Cancer of endometrium Cancer of prostatic glands Blood cancer
Methionine synthase reductase (MSR)	66A → G	Blood cancer Cancer of inflammatory bowel syndrome Cancer of gastric cells
Methionine synthase (MTR)	2756A → G	Cancer of head and neck Cancer of inflammatory bowel syndrome Lung cancer
Methylenetetrahydrofolate dehydrogenase (MTHFD)	1958G → A 401G → A	Blood cancer Cancer of ovaries Cancer of gastric cells
Betaine-homocysteine methyltransferase (BHMT)	742G → A	Cancer of breast Cancer of squamous cell Uterine carcinoma
TCN2	776 G → C	Brain tumor Blood cancer Cancer of ovaries
TYMS	TS 3′-UTR TSER	Cancer of stomach Oral cancer Cancer of breast
Cystathionine beta-synthase	595G → A	Cancer of liver

4. 4. Hyperhomocysteinemia and oxidative stress

Many cellular mechanisms have been proposed and confirmed Homocysteine has been shown to cause apoptosis and lipid formation in vitro and in HHcy induced atherogenesis in vivo, including induction of inflammation and oxidative stress **(Zhou and Austin, 2008**). Further, it has been shown that hyperhomocysteinemia impairs the antioxidant system, which is in responsible of neutralizing ROS and preventing oxidative stress. It has been shown that homocysteine decreases the activity of the antioxidant enzymes catalase, glutathione peroxidase, and superoxide dismutase, causing an accumulation of ROS and oxidative stress (**Bischoff-Ferrari *et al.*, 2009**). Hcy elevated levels of H2O2, ONOO, and O2 in a time and concentration dependent manner, and it also caused cytotoxicity (Figure 14) (**Yan *et al.*, 2006**).

Also, Hcy has the ability to inhibit the synthesis of antioxidant enzymes like glutathione peroxidase (GSH-Px), which might destroy the toxic effects of reactive oxygen species (ROS) **(Upchurch *et al.*, 1997)**.

According to certain studies, Hcy may promote the production of hydroxyl radicals that start lipid peroxidation through Hcy auto-oxidation and the synthesis of thiolactone. It has been proposed that free radicals and lipid peroxidation products like malondialdehyde (MDA) and 4-hydroxy-2- nonenal (HNE) , which are major end products of lipid peroxidation, induced damage to proteins and carbohydrate (**Cavalca *et al.*, 2001**). NADPH oxidase is an enzyme complex that produces ROS, which are extremely reactive molecules that can cause cellular damage, it has been demonstrated that hyperhomocysteinemia activates NADPH oxidase, leading to an increase in ROS production and oxidative stresss (**Zhao and Mooney, 2010**). According to the results of (**Tyagi, 2005**), Hcy activates PAR-4, which causes the production of reactive oxygen species by raising NADPH oxidase and lowering thioredoxin expression and reducing NO bioavailability in cultured MVEC, increasing NO2-tyrosine formation and accumulating ADMA by decreasing.

Moreover, it has been discovered that elevated homocysteine levels can activate pro-inflammatory pathways, producing cytokines and other pro-inflammatory mediators that can further contribute to oxidative stress (**Chen and Li, 2010**).

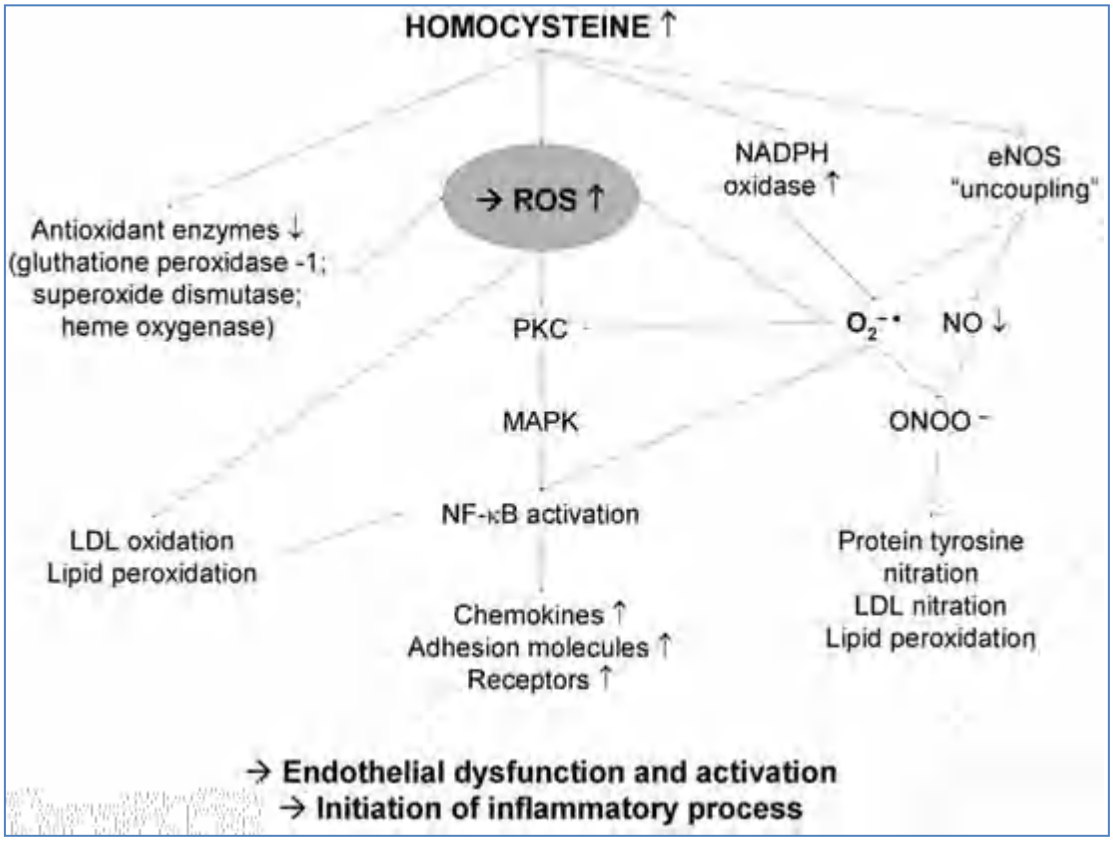

Figure 15. Homocysteine increases reactive oxygen species in endothelial cells, which induce endothelial dysfunction and initiate inflammatory processes (**Papatheodorou and Weiss, 2007**).

5. Antioxidant activity

5.1. Introduction

Evaluating the antioxidant activity/capacity levels of biological fluids and foods is performed in clinical biochemistry for the diagnosis and treatment of oxidative stress-related disorders, for meaningful comparison of foods in terms of antioxidant content, and for controlling variances within or between products (**Apak *et al.*, 2016**). Many assays with various methods can be used to monitor antioxidant activity **(Shahidi and Ying, 2015).**

5.2. Free radicals

A free radical is any species that has one or more unpaired electrons and can exist independently (**Halliwell *et al.*, 1995**). We distinguish the primary radicals, which derive from oxygen by reductions of one electron, for example: the superoxide anion (O2 •-), nitric oxide (NO•), hydroxyl radical (•OH), alkoxyl radical (RO•) and the peroxyl radical (ROO•), and the secondary free radicals that are produced when primary free radicals react on biochemical compounds of the cell; examples

of these include: hydrogen peroxide (H2O2), singlet oxygen (1O2), and nitroperoxide (ONOOH) (**Yoshikawa *et al.*, 2000**).

Free radicals and reactive oxygen species (ROS) have been linked to the etiology and/or development of a variety of illnesses, and in aging. Several proteins that have been oxidatively changed by free radicals include side-chain carbonyl derivatives that can be utilized as markers for protein oxidation (**Moskovitz *et al.*, 2002**).

Table 04: Examples of free radicals (**Halliwell *et al.*, 1994**).

Name	Formula	Comments
Hydrogen atom	$H^{\cdot}$	The simplest free radical.
Trichloromethyl	$CCl_3^{\cdot}$	A carbon-centered radical (i.e., the unpaired electron resides on carbon). $CCl_3^{\cdot}$ is formed during metabolism of CCl_4 in the liver and contributes to the toxic effects of this solvent.[5] Carbon-centered radicals usually react fast with O_2 to make peroxyl radicals, e.g., $CCl_3^{\cdot} + O_2 \rightarrow CCl_3O_2^{\cdot}$.
Superoxide	$O_2^{\cdot -}$	An oxygen-centered radical. Selectively reactive.
Hydroxyl	$OH^{\cdot}$	A highly reactive oxygen-centered radical. Attacks all molecules in the human body.
Thiyl	$RS^{\cdot}$	A group of radicals with an unpaired electron residing on sulfur.
Peroxyl, alkoxyl	$RO_2^{\cdot}$, $RO^{\cdot}$	Oxygen-centered radicals formed (among other routes) during the breakdown of organic peroxides.
Oxides of nitrogen	$NO^{\cdot}$, $NO_2^{\cdot}$	Nitric oxide is formed in vivo from the amino acid L-arginine.[6] Nitrogen dioxide is made when $NO^{\cdot}$ reacts with O_2 and is found in polluted air and smoke from burning organic materials, e.g., cigarette smoke.[7]

5.3. Definition of an antioxidant

Antioxidants are substances that, when present in extremely low quantities in food or the body, delay, control, or prevent oxidative processes that lead to food quality deterioration or the beginning and development of degenerative illnesses in the body. This definition of antioxidants includes free radical scavengers, singlet oxygen quenchers, inactivators of peroxides and other reactive oxygen species (ROS), metal ion chelators, quenchers of secondary oxidation products and inhibitors of pro-oxidative enzymes (**Shahidi and Zhong, 2007**).

5.4. Antioxidants division

There are two main groups of antioxidants: natural and synthetic. (**Zehiroglu and Ozturk Sarikaya, 2019**), the third group is known as nature-identical antioxidants (**Pokorný, 2007**).

Antioxidants can also be categorized as endogenous and exogenous depending on their sources, enzymatic or non-enzymatic depending on their effects, and water-soluble or lipid-soluble depending on their solubility (**Gulcin, 2012**), this classification is given in the tables below with their samples (**Table 06**).

5.4.1. Naturally occurring antioxidants

Natural antioxidants are most frequently encountered in plants (**Pokorný, 2007**) are listed in (**Table 5)**, antioxidants can exist naturally in animals and microorganisms. Higher plants and their components are a rich source of natural antioxidants such as tocopherols and polyphenols. Additionally, antioxidants from marine sources, such as those found in algae, fish and shellfish, and marine bacteria, have also been considered (**Amarowicz *et al.*, 1999**).
The most important natural antioxidants are vitamin E (tocopherol), carotenoids (β-carotene), vitamin C (ascorbic acid), polyphenols especially flavonoids (**Labed *et al.*, 2016**).

The most significant polyphenols in plants are flavonoids, phenolic acids, and stilbenes, which have demonstrated a variety of beneficial bioactivities, including anticarcinogenic characteristics, chemical structures can range from very simple molecules to very complex molecules (**Jakobek, 2015**), flavonoids are essential antioxidants because of their high redox potential, which lets them function as reducing agents, hydrogen donors, and singlet oxygen quenchers. Moreover, they have the potential to chelate metal (**Ignat *et al.*, 2011**). Phenolic acids account for almost one-third of the dietary phenols present in plants, both free and bound, and are recognized to be powerful antioxidants. Relieve almost all oxidant molecules, such as free radicals, by hydroxyl groups (**Ignat *et al.*, 2011; Sevgi *et al.*, 2015**).

The most important vitamins as natural antioxidants are vitamins E and C. (**Zehiroglu and Ozturk Sarikaya, 2019**), Vitamin E is a chiral molecule that contains eight different stereoisomers (a, b, c, d tocopherol and a, b, c, d tocotrienol), in humans, only a-tocopherol is the most bioactive (**Pham-Huy *et al.*, 2008**), Vitamin C, which comprises ascorbic acid and its oxidation product dehydroascorbic acid, has several biological activities in the human body (**Zehiroglu and Ozturk Sarikaya, 2019**).

Carotenoids (carotenes and xanthophylls) are non-enzymatic natural antioxidants (**Podsedek, 2007**). The antioxidant effects of carotenoids actions depend on their singlet oxygen quenching properties and their ability to capture peroxyl radicals (**Zehiroglu and Ozturk Sarikaya, 2019**).

Table 05: The most frequently encountered natural antioxidants in plants (**Pokorný, 2007**).

Antioxidant class	Examples of substances
Phenolic acids	
Hydroxybenzoic acid series	Vanillic acid
Hydroxycinnamic acid series	Ferulic acid, chlorogenic acid
Flavonoids	Quercetin, catechin, rutin
Anthocyanins	Delphinidin
Tannins	Procyanidin, ellegic acid, tannic acid
Lignans	Sesamol
Stilbenes	Resveratrol
Coumarines	*ortho*-Coumarine
Essential oils	*S*-Carvone

5.4.2. Synthetic antioxidants

The synthesis or biosynthesis of antioxidants by a human with expertise in the industry is another method of producing antioxidants. They have been produced and developed for the benefit of humanity; In order to protect consumers from potential health dangers, they have tested extremely complex and costly studies to determine their safety, in addition to protecting the producers from any consumers complaints (**Pokorný, 2007**).

5.4.3. Nature-identical antioxidants

The third category of substances contains antioxidants that are identical to natural antioxidants present in foods, but synthesized in the industry. In contrast to natural antioxidants they are pure substances, inexpensive, readily accessible, and have reproducible properties, including antioxidant activity. Thus, they combine the advantages of the natural and the synthetic antioyxidants (**Pokorný, 2007**).

Table 06: Classification of antioxidants based on their origin (**Zehiroglu and Ozturk Sarikaya, 2019).**

Antioxidants: based on their origin				
Natural				Synthetic
Enzymatic	Non-enzymatic			BHA (Butylated hydroxytoluene), BHT (Butylated hydroxyanisole), Trolox, TBHQ (Tertiary-butyl hydroquinone)
Primary enzymes	Other antioxidants	Vitamins		
Super oxide dismutase, catalase, glutathione peroxidase	Protein	Vitamin A, Vitamin C, Vitamin E		
Secondary enzymes	Albumin, ceruloplasmin, lactoferrin, transferrin	Minerals		
Glutathione reductase, glutathione 6-phosphate dehydrogenase	Non protein	Selenium, zinc, cooper, iron		
	Bilirubin, Ubiquinol, Uric acid	Carotenoids		
	Cofactors	β-carotene, lycopene, lutein, zeaxanthin		
	Coenzyme Q_{10}	Polyphenols		
		Flavonoids	Phenolic acid	
		Flavonols	Hydroxy-cinnamic acids	
		Quersetin, kaempherol	Ferulic, p-coumaric	
		Flavanols	Hydroxy-benzoic acids	
		Catechin	Gallic acid, ellagic acid	
		Flavones		
		Chrysin		
		Flavanones		
		Hesperidin		
		Isoflavanoids		
		Genistein		
		Anthocyanidins		
		Cyanidin, pelagonidin		
Endogenous		Exogenous		

5.5. Methods for determining antioxidant activity

There are several assays available to evaluate the total antioxidant activity of body fluids, food extracts, and pure compounds. Each technique associate with the generation of a different radical that acts via a variety of mechanisms, as well as the measurement of a range of end points at a fixed time point or over a range (**Re *et al*; 1999**); According to their mechanisms, they may be divided into two categories: either by the transfer hydrogen atom, or by the transfer of a single electron (**Sanchez-Moreno, 2002**).

The first category measures the capacity of antioxidants against inhibiting oxidation reactions in a model system by monitoring the association changes using chemical, physical, or instrumental means. Methods for conducting radical scavenging experiments include those based on electron transfer (ET) or hydrogen atom transfer (HAT) mechanisms. Oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP) and crocin bleaching assays are the main methods that measure HAT while Trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP), and DPPH assays represent ET-based methods (**Shahidi and Ying, 2015)**.

The most used tests are:

- ✓ The 2,2-Azino-Bis-3-ethylbenzoThiazoline-6-Sulfonic acid test (ABTS).
- ✓ The 1,1-diphenyl 2-picrylhydrazyl (DPPH) test.
- ✓ The test using the reducing power of ferric ions, Ferric Reducing Antioxidant Power (FRAP).
- ✓ The test using the reducing power of cupric ions, CUPric Reducing Antioxidant Capacity (CUPRAC).

6. Species *Astragalus armatus*

6.1 Classification

KINGDOM : Plantae
PHYLUM : Tracheophyta
CLASS : Magnoliopsida
ORDER : Fabales
FAMILY : Fabaceae
GENUS : *Astragalus*
SPECIES : *Astragalus armatus* Willd

6.2. Botanical description:

Astragalus armatus Willd, according to Quezel and Santa (**Quezel and Santa, 1963**), is a plant with pods remaining enclosed in the calyx that is highly accrescent, vesicular papery, and glabrous reticulate. Shrub 20-50 cm tall with more or less diffuse stems with rachis of indurant leaves 3-8 pairs of leaflets that are rapidly deciduous, white flowers that are pinkish. Its colloquial name is « Gdad », « El Guendou » and « Chouk edderban » (Figure 16).

Figure 16. *Astragalus armatus* Willd.

6.3. Geographical distribution

The species *Astragalus armatus* growing in North Africa. It is an endemic species, it is found in Algeria, Morocco and Tunisia.

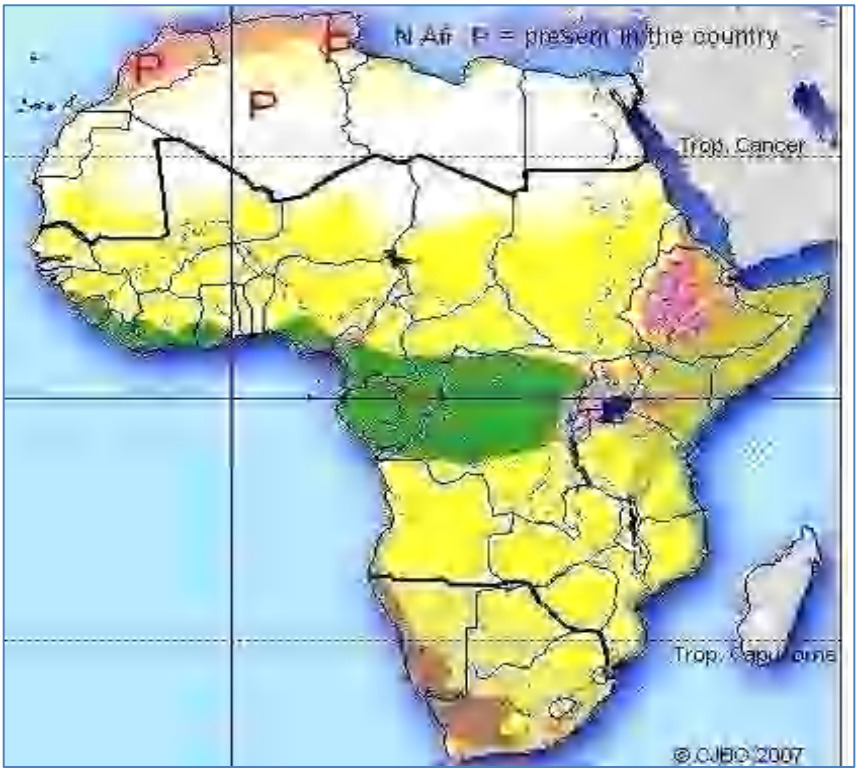

Figure 17. Geographic distribution of the species *Astragalus armatus* Willd (**Greuter *et al.*, 1989**).

6.4. Use in traditional medicine

It is used in traditional medicine, known as "Guendoul," to cure a variety of illnesses (**Trabut, 1935**). For instance, in Ghardaia, the bark and seeds are frequently used to treat many types of wounds as well as stomach problems, pain, fever, and constipation (**Voisin, 1987**). In southern Morocco, *A. armatus* fresh ground aerial parts are used to cure snake and scorpion stings (topical application) (**El Rhaffari and Zaid, 2002**).

CHAPTER II

Experimental studies

I. Materials and methods

1. Plant extract

Aerial parts of *A. armatus* were collected from Bekira-Constantine (East of Algerian) in May 2007. The voucher specimen was kept in the Herbarium of the Faculty of Sciences (University of Constantine-1) under the number LOST (Aa.05.07) (**Khalfallah *et al*., 2014**). Extraction of the aerial part was carried out at the obtention laboratory of therapeutic substances (LOST) of the University Constantine-1.

2. Extraction

Air-dried powdered aerial parts of Astragalus armatus (1.5 kg) were macerated four times with 70% EtOH solution. The hydro-alcoholic solution was concentrated under reduced pressure to dryness and the residue was dissolved in water and kept in overnight. After filtration, the aqueous solution was successively extracted with CH2Cl2, EtOAc and n-BuOH for three times with each solvent, then the EtOAc and n-BuOH extracts were concentrated to dryness (**Khalfallah *et al*., 2014**).

3. Chemicals material

The Chemical products used during our study are:

L-methionine, phosphate buffer saline (PBS), chloroform, formalin 10%, solution of bouin , coomassie brilliant blue, dithiobis-2-nitrobenzoic acid (DTNB), orthophosphoric acid (85%), ethylene di-amine, tris, tetra acetic acid (EDTA, 0.02M), ethanol of different concentrations (50%, 70% and 96%), bovine serum albumin (BSA), sulfo-salcylic acid (0.01M), Nacl, Hcl, NaoH, butanol, xylene, glycerin and paraffin.

The concentration of homocysteine in plasma was determined by the Immulit homocysteine Kit (Siemens, Finland), other used chemicals were obtained from the chemical company Sigma.

4. Equipment

We used the following equipment:

Precision Weighing Balances (readability 0.01g) to determine the weight of mice, Precision Weighing Balances (readability 0.00001g) to weigh methionine and plant extract, heating magnetic stirrer, pH meter, centrifuge, oven, microtome, Spectrophotometer, Photo microscope connected to computer.

5. Animal material

The research was performed on twenty eight healthy adult male *Mus Musculus* mice, 2.5 to 3 months old, weighing between 30 and 35 g. All animals were obtained from the Central Pharmacy of Algeria and housed in plastic cages with free access to water and diets for 21 days with experimental diet control (the composition of the food is shown in table 02). Mice were divided into four groups of similar mean body weights and conducted under standard laboratory conditions of humidity, temperature and light. They received tap water and a standard laboratory diet purchased from EL REGHAIA feed Co. (Algiers, Algeria).

Table 07: Composition of diet taken by the mice during 21 days (L'ONAB nutrition)

Composition	**Amount in (g / kg)**	**Percentage (%)**
Corn	620	62
Soja	260	26
Phosphate	16	1,6
Limestone	9	0,9
Cellulose	10	1
Minerals	10	1
Vitamins	10	1

6. *In vivo* experimental study

6 .1. Experimental treatments

After an acclimatization period of one week, the pubertal mice were randomly assigned into 4 groups (7 mice per group):

- The control group (F) was fed with white flour (0.50 mg/mouse).
- The group (M) was fed with L-methionine (400 mg/kg/day).
- The group (MP) received L-methionine (400 mg/kg/day) plus A. armatus extract (100 mg/kg/day).
- The group (P) was treated with *A. armatus* extract (100 mg/kg/day).

Throughout the treatment period (21 days), the weights and diet consumption of mice were taken daily throughout the experiment at the same time (**Zerizer and Naimi, 2004**).

The dose of each treatment is calculated according to the average live weight of the mice of each cage and given to the mouse once a day, L-mehionine and A. armatus extract were orally administered to mice with white flour (0.50 mg/mouse) and given free access to food and water.

Table 08: Treatment of mice

Experimental Group	**Substance administered**	**Number of animal**	**Daily dose**	**Duration of the experiment**
F	Flour	7	0.50 mg/mouse	21 days
M	Flour + L-methionine	7	400 mg/kg/day	21 days
MP	Flour + L-methionine + *A. armatus* extract	7	400mg/kg/day + 100 mg/kg/day	21 days
P	Flour + *A. armatus* extract	7	100 mg/kg/day	21 days

6.2. Blood and tissue sampling

After 21 days of feeding, animals have fasted overnight and before sacrificing animals, blood samples were taken from the retro-orbital plexus into EDTA tubes and centrifuged, the plasma was stored at -20˚C until biochemical analysis. The aorta, heart and liver were immediately removed and washed with PBS saline, a fraction of liver of each animal was used to determine biochemical parameters while other fractions of aorta, liver and heart of each animal were used for histological examination.

6. 3. Biochemical analysis

6. 3. 1. Measurement of plasma Hcy levels, lipids status

The determination of the plasma Hcy and lipids status was carried out at the IBN SINA medical analysis laboratory in Constantine.

6. 3. 1. 1. Plasma Hcy determination

The determination of total Hcy was done by an IMMULITE analyzer, the principle is based on a competitive chemiluminescence enzyme immunoassay technique in solid phase.

6. 3. 1. 2. Lipids determination

The lipid assay was performed using an (INTEGRA 400 type auto-analyzer), using an enzymatic colorimetric method. A complete lipid panel will include the determination, after twelve hours of fasting, of total cholesterol, triglycerides, LDL and HDL.

- **Dosage of total cholesterol**

Cholesterol is an unsaturated alcohol present in blood, bile, and brain tissue. In addition to other tissues, the liver and intestinal wall are two places where it is primarily synthesized. It functions as a precursor of bile acids, adrenal and gonadal steroid hormones, as well as vitamin D (**Cox and Garcia-Palmieri, 1990**).

The series of reactions involved in the assay system are as follows:

$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{Cholesterol Oxidase}} \text{Cholesterol} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol Oxidase}} \text{Cholest-4-ene-3-one} + H_2O_2$$

$$2H_2O_2 + \text{4-AAP} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + 4\,H_2O$$

The intensity of quinone-imine colouration, measured at 500 – 550 nm, is proportional to the amount of total cholesterol present in the serum sample (**Young, 2001**).

The formulas below can be used to calculate the cholesterol concentration.

$$\text{Cholesterol Concentration} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}} \text{ (Cholesterol standard)*200 mg/dl (mmol/L)}$$

➢ Dosage of Triglycerides

The Triglycerides are determined after enzymatic hydrolysis by lipases. The indicator is a quinone-imine formed from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol, the reaction being catalyzed by peroxidase. Absorbance is measured at a wavelength of 505 nm (**Young, 2001**).

$$\text{Triglycerides (TG)} \xrightarrow{\text{Lipoprotein Lipase (LP)}} \text{Glycerol+ fatty acids}$$

$$\text{Glycerol +ATP} \xrightarrow{\text{Glycerol Kinase (GK)}} \text{Glycerol-3-phosphate (G3P) + ADP}$$

$$\text{G3P + O2} \xrightarrow{\text{Glycerol Phosphate Oxidase (GPO)}} \text{Dihydroxyacetone phosphate (DAP) + 2 H2O2}$$

$$\text{2 H2O2+ 4-AAP + 4-Chlorophenol} \xrightarrow{\text{Peroxidase (POD)}} \text{Quinoneimine dye + 4 H2O}$$

the formulas below can be used to calculate the cholesterol concentration.

$$\text{TG concentration} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}} * 200 \text{ (Standard concentration)} = \text{mg/ml} * 0.0114 \text{ mmol/L.}$$

➢ Dosage of HDL-c

The principle consists in selectively precipitating the lipoproteins which contain apoB (LDL and VLDL) by phosphotungstate in the presence of magnesium ion, and after centrifugation, HDL cholesterol is determined in the supernatant by the same enzyme technique as the total cholesterol (**Young, 2001**), and the calculation as shown below:

$$\text{HDL-c Concentration} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}} (\text{Standard concentration}) * 200 \text{ mg/dL}$$

- **Dosage of LDL-c**

LDL cholesterol is obtained by direct calculation according to the Fiedewald formula

LDL = total cholesterol - HDL - triglycerides / 5

When the TG level is greater than 3.4 g/l (3.75 mmol/l), LDL cholesterol cannot be calculated by this formula, so it must be dosed by direct enzymatic method.

6. 3. 1. 3. Determination of oxidative stress parameters

- **Preparation of the homogenate**

For the determination of oxidative stress parameters of GSH and CAT activities, the liver (0.5 g) was homogenized in 2 ml of TBS solution (Tris 50 mM, NaCl 150 mM, pH 7.4); the homogenates were centrifuged at 9000 ×g for 15 min at 4˚C. After that, the pellet was discarded and the supernatant was subjected to biochemical assays.

- **Protein determination**

Protein concentrations concentration was measured by the method of Bradford (1976), using bovine serum albumin as standard. The procedure is based on the formation of a blue complex between the comaissie bruillant blue G-250 dye, and proteins in solution. The amount of absorption is proportional to the protein present.

Liver homogenate sample 0.1ml was mixed with 5ml Bradford reagent and was allowed stand for 5min. Then the absorbance was measured at 595 nm using a spectrophotometer by comparing to the blank reaction.

The protein concentration of a test sample is determined by comparison to that of a standard series of bovine serum albumin to reproducibly exhibit a linear absorbance profile in this assay (Figure 01 annex).

➢ Determination of glutathione reduced

The concentration of the glutathione reduced in the liver was determined according by the method of Weckbecker and Cory (**Weckbercker and Cory, 1988**). The spectrophotometric reader assay method for GSH involves oxidation of GSH by the sulfhydryl reagent 5,5′-dithio-bis2-nitrobenzoic acid (DTNB) to form the yellow derivative 5′-thio-2-nitrobenzoic acid (TNB), measurable at 412 nm.

For the the glutathione dosage, the following experimental protocol was used:

Liver homogenate sample (0.8ml) was deproteinized with (0.2ml) of 5-sulfosalicylic acid solution (0.25%) and was allowed stand on ice for 10 min. Following centrifugation at 1000 tours/mn) during 5minutes to remove the precipitated protein. (0.5 ml) of supernatant was mixed with 1 ml Tris/EDTA buffer (pH 9.6) and (0.025 ml) of DTNB-reagent (0.01M 5,5'-dithiobis-2-nitrobenzoic acid) and left at room temperature for 5 min. Then the absorption was measured at 412 nm using a spectrophotometer by comparing to the blank reaction.

The glutathione concentration was calculated by the following formula:

$$\text{GSH (nmol/mg of protein)} = \frac{OD \times 1 \times 1.525}{13100 \times 0.8 \times 0.5.\text{mg protein}}$$

- OD: optical density
- 1: total volume of solutions in the deproteinisation (0.8ml homogenate+ 0.2ml 5-sulfosalicylic acid)
- 1.525: total volume of the solutions used in the assay of GSH (0.5ml supernatant+ 1 ml Tris/EDTA+ 0.025 ml DTNB)
- 13100: absorbance coefficient at Groupment—SH to 412nm
- 0.8: volume of homogenat sample
- 0.5: volume of supernatant

➢ Determination of catalase

Catalase is a common enzyme found in nearly all living organisms exposed to oxygen. It catalyzes the decomposition of hydrogen peroxide (H2O2) to water and oxygen. Catalase is a tetramer of four polypeptide, It contains four porphyrin heme (iron) groups that allow the enzyme to react with the

hydrogen peroxide. It was estimated in the liver homogenate in a UV spectrophotometer as described by Aebi (1984). The specific activity of catalase has been expressed as mmol of H2O2 consumed/min/ mg protein. The difference in absorbance at 240 nm per unit time is a measure of catalase activity.The reaction is thought to happen in two stages:

Catalase-(Fe III) + H2O2 ⟶ [Catalase-H 2O-(Fe V)] + H2O

[Catalase-H 2O-(Fe V)] + H 2O2 ⟶ Catalase-(Fe III) + H2O + O2

2 H2O2 ⟶ 2 H2O + O2

Table 09: The concentrations and amounts of reagents needed for the dosage of catalase activity.

Reagents	**Sample (µl)**	**blank (µl)**
Phosphate buffer (100Mm, PH7.5)	790	800
H2O2 (500Mm)	200	200
Sq (1 to 1.5 mg prt/ml)	10	0

Sq: supernatant quantity

The activity of catalase was estimated by the decrease of absorbance at 240 nm for 1 min (15 and 60 seconds) as a consequence of H2O2 consumption.

The following formulas were directly calculated to determine the catalase activity:

$$\text{Catalase activity (mmol H2O2/min/ mg prot)} = \frac{\Delta DO}{\varepsilon \times L \times \chi \times Fd}$$

ε: extinction coefficient (= 0.043 mM-1.cm -1).

L: The length of the cuvette used (1 cm).

χ: protein quantity mg/ml.

Fd: 0.02 (dilution factor of the H2O2 in the buffer).

λ: 240 nm.

6. 4. Histological analysis

Following the collection of blood samples, the animals were sacrificed, and samples from the aorta, heart, and liver were collected for light microscopic examinations.

- The samples were rinsed of all adherent tissues with phosphate buffered saline (0.9%). After that,they are stored in little vials with diluted formol 10%;
- Inclusion of the different parts of the aorta in alcoholic Bouin solution (appendix) for 5 min for staining (because of their transparent color);
- Dehydration in three different concentrations of ethanol (50%, 75% and 96%), the duration for each concentration is 1h and 30 min (the bath is changed every 30 min), then in butanol for three days;
- Clarification with xylene for 20 min (the bath is changed every 10 min);
- Inclusion in paraffin: three paraffin baths (the duration of each bath: 1h and 30min, the oven temperature is maintained at (60°C);
- Production of paraffin blocks containing the sample to be studied;
- Realization of 3µm thick sections using a microtome (Leica RM 2135);
- The sections obtained are placed in a tidal bath (50°C), spread on slides, then dried on a hot plate (50°C) overnight; the coloration of the slices was done at Niha laboratory, Annaba;
- Deparaffinization of slides in xylene for two hours, then air drying for two more hours;
- The photos of sections were taken by photo-microscope.

6. 5. Statistical analysis

The biochemical analyzes (Hcy, lipids and antioxidant enzyme activities) are presented in the form of means accompanied by the standard error (mean ± SEM). The comparison between the groups was performed by the One-Way ANOVA test, when a significant difference is observed; Tukey's multiple comparison test is performed. All analyses related to the case–control study were performed using the Statistical Package for the Social Sciences (SPSS software version 20). The comparison or correlation is considered, according to the probability (P), as:

- No significant if $P > 0.05$.
- Significant (*) if $P < 0.05$.
- Highly significant (**) if $P < 0.01$.
- Very highly significant (***) $P < 0.001$.

7. In *vitro* experimental study

7. 1. Evaluation of antioxidant activity of n-butanol extract of *A. armatus*

7. 1. 1. Total antioxidant activity

The activities of the sample solutions (50-500 µg mL-1) were evaluated following the phosphomolybdenum method (**Prieto *et al.*, 1999**) based on the reduction of Mo (VI)to Mo (V) by antioxidants and the subsequent formation of specific green phosphate/Mo (V) compounds at acidic pH . The extract solution (0.3 mL) was added to (2.7 mL) of the reagent solution [(4 mM) ammonium molybdate, (0.6 M) sulfuric acid, (28 mM) sodium phosphate]. The reaction was incubated at 95 °C during 90 minutes, and then cooled to room temperature. The absorbance of the resulting green sample phosphomolybdenum complex was read at 695 nm (**Ozen *et al.*, 2017**). BHA, BHT and ascorbic acid were used as positive controls.

7. 1. 2. Reducing power assay

In this assay, the reductants antioxidant causes the reduction of the Fe+3/ ferricyanide complex to the Fe2+ form and monitored by measuring the absorbance at 700 nm (**Oyaizu *et al.*, 1986**). 1 mL of the sample solutions (50-500 µg/mL) were added to 2.5 mL phosphate buffer (pH 6.6, 0.2 M) and potassium ferricyanide (1%; 2.5 mL), respectively. After incubating for 25 min at 50 °C, tricholoroacetic acid TCA (10%; 2.5 mL) was added to the mixture and centrifuged at 3.000 x g. Finally, the supernatant mixture (2.5 mL) was combined with (0.5 mL; 0.1 %) ferric chloride FeCl3 solution and the absorbance was read at 700 nm. It is indicated that the high absorbance of the sample has effective reducing power in the reaction condition that the reducing capacity had increased. BHA, BHT, trolox and ascorbic acid were used as positive controls.

7. 1. 3. Free radical scavenging assay (DPPH˙)

The electron donation activities of the sample solutions was measured spectrophotometrically by bleaching of the purple-colored solution of free radical scavenging activity according to the technique available in report with a slight modification (**Blois, 1958**). The DPPH· solution (1 mL) was mixed with (1mL) of sample solutins (50-500 µg mL-1). After incubating in the dark at room for 30 min, the absorbance of mixture was monitored at 517 nm. The reduction of sample absorbance indicated higher DPPH˙ scavenging activity (**Ozen *et al.*, 2011**). BHA, BHT, trolox and ascorbic acid were used as positive controls.

The activities were calculated as percentage of DPPH· discoloration, using the equation:

Free radical scavenging activity % = [(A (blank) - A (sample))/ A (blank)] ×100.

7. 1. 4. Metal chelating assay

The metal chelating activity of the sample was assessed as described by **Dinis *et al*. (1994**) using EDTA as a positive control. In the presence of other chelating compounds, this complex is disrupted with chelator compounds. Briefly, the different concentrations (50-500 µg/mL) of the sample were mixed separately with FeCl2 (2 mM, 0.05 mL) solution. After incubation at 25 °C for 5 min, the reaction was initiated by the addition of ferrozine (5 mM; 0.3 mL). Each mixture was shaken vigorously and left to stand at 25 °C pending 10 min. The absorbance of the mixture was read at 562 nm. The activities were calculated as percentage of inhibition of ferrozine-Fe^{+2} complex formation according to formula:

Metal chelating activity % = [(A0-A1)/A0] x 100.

Where A0 is the absorbance of the ferrozine-Fe^{+2} complex (control) and A1 is the absorbance of the test solutions.

7. 2. Evaluation of antiproliferative activity of n-butanol extract of *A. armatus*

7. 2. 1. Culture cell preparation

HeLa (human cervix carcinoma) and C6 (rat brain tumor) cells were developed in DMEM-HG (Dulbecco's modified eagle's medium) with 10% (v/v) FBS (fetal bovine serum) and 2 % Penicilin/streptomycin solution, at 37 °C in a humidified atmosphere of 5 % CO2 at Plant Research Laboratory, Department of Chemistry, University of Cankiri Karatekin, Turkey.

HeLa and C6 cells were detached from bottom of the culture flask using 10 mL Trypsin- EDTA mixture. After detaching, the same volume of culture medium was placed in the flask and mixed thorougly and put in centrifuge 600 tours during 5 min (Nüve NF 800, Turkey). 5mL of medium was mixed carefully with the supernatant. The concentration of cells in this suspension was determined using CEDEX HIRES Cell Counter (**Demirtas and Sahin Yaglioglu, 2012).**

7. 2. 2. Cell proliferation assays

Cells were plated in 96-well culture plates (COSTAR, Corning, USA) at a densitiy of 30.000 cells per well. The activities of samples were investigated on 100, 75, 50, 40, 30, 20, 10, and 5 µg/mL. 5-

FU was used as standard compound. The cells were then incubated overnight before applying the BrdU Cell Proliferation ELISA assay reagent (Roche, Germany) according to the manufacturer's procedure.

The amount of cell proliferation was assessed by determining the absorbance (A) at 450 nm of the culture media by using a microplate reader (Awareness Chromate, USA). Results were reported as percentage of the inhibition of cell proliferation, where the optical density measured from vehicle-treated cells was considered to be 100% of proliferation. All assays were repeated at least twice using against HeLa and C6 cells.

Percentage of inhibition of cell proliferation was calculated as follows:

$$\% = [1-(A\text{treatments} / A\text{vehicle control})] \times 100$$

The stock solution of the extracts were prepared in dimethyle sulfoxide (DMSO) and diluted with Dulbecco's modified eagle's medium (DMEM; 1:20). DMSO final concentration is below 0.1% in all tests.

IC50 and IC75 values were determined using the ED50 plus v1.0 programs. The results of investigation *in vitro* are means ± SD of six measurements. Differences between groups were tested with ANOVA. P values of <0.01 were considered as significant and analyzed by SPSS (version 11.5 for Windows 2000) (**Sahin Yaglioglu *et al*., 2014**).

Results

1. Tests *in vivo*

1.2. Plasma Hcy concentration

The concentration of homocysteine were (8.686 ± 1.893 µmol/l) in F group, (7. 22 ±1.365 µmol/l) in P group, (13.48 ± 3.713) in M group and (9.494 ±3.537 µmol/l) in MP group; these values were statistically different significantly (P=0.018) between groups. The Tukey test revealed a significant difference between the group (M and P) P = 0.014 (Figure 18).

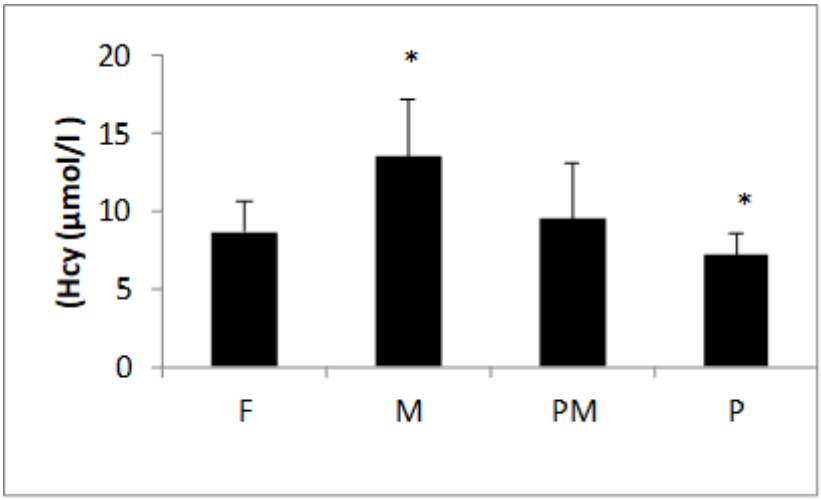

Figure 18. The interaction of L-methionine and *A. armatus* extract on Hcy in mice during 21 days of treatment, (F) control group, (M) treated with L-methionine, (PM) treated with L-methionine and *A. armatus* extract, (P) treated with *A. armatus* extract. Values are the means±SEM (n); *p<0.05.

1.2. Lipids status

1.2.1. T-cholesterol

The results of the determination of total cholesterol in the F group (0.994 ±0.31 g / l), P group (1.19±0.20 g / l), M group (1.074 ± 0.13 g / l), MP group(0.938± 0.08 g / l) show that there is a difference between groups but not significantly (P> 0.05) (Figure 19).

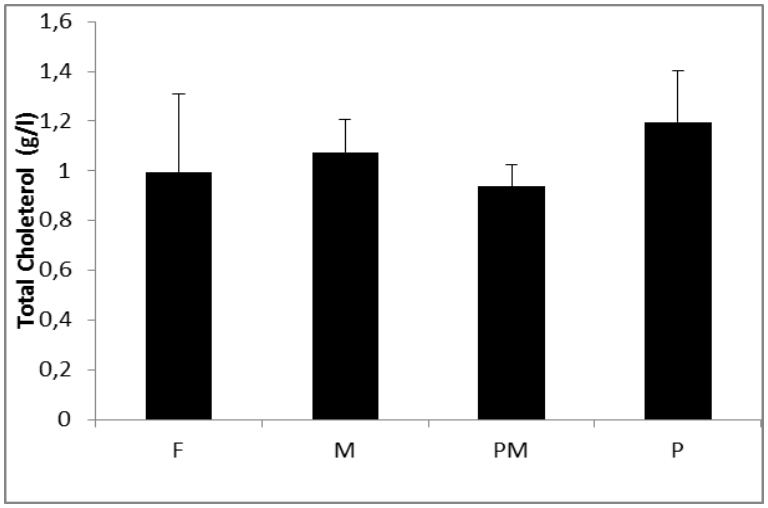

Figure 19. The interaction of L-methionine and *A. armatus* extract on Tch in mice, during 21 days of treatment, (F) control group, (M) treated with L-methionine, (PM) treated with L-methionine and *A. armatus* extract, (P) treated with *A. armatus* extract. Values are the means±SEM (n); *p<0.05.

1.2.2. LDL-c

The results of the determination of LDL-c in F group (0.18±0.07g/l), P group(0.53±0.33 g/l) M group (0.65±0.20 g / l), MP group (0.37±0.16 g/l) show that there is a highly and significantly difference between groups (P = 0.008). The Tukey test revealed a significant difference between the groups (F and M) P= 0.004) (Figure 20).

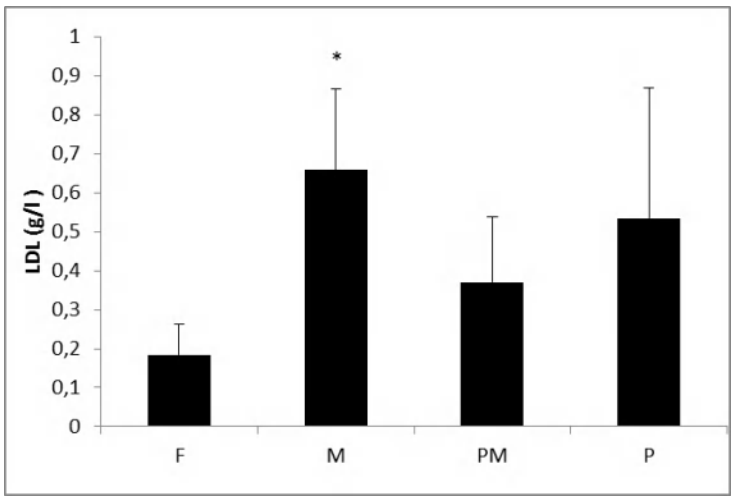

Figure 20. The interaction of L-methionine and *A. armatus* extract on LDL in mice, during 21 days of treatment, (F) control group, (M) treated with L-methionine, (PM) treated with L-methionine and *A. armatus* extract, (P) treated with *A. armatus* extract. Values are the means±SEM (n); *p<0.05.

1.2.3. HDL-c

The results of the determination of HDL-c in F group (0.80±0.09 g/l), P group (0.66 ±0.46g/l) M group (0.75±0.18 g/l), MP group (0.66±0.43 g/l) show that there is a difference between groups but not significantly (P> 0.05) (Figure 21).

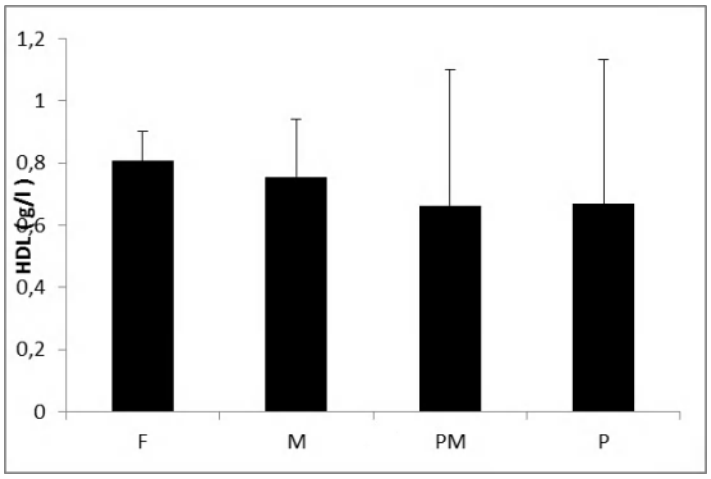

Figure 21. The interaction of L-methionine and *A. armatus* extract on HDL in mice, during 21 days of treatment, (F) control group, (M) treated with L-methionine, (PM) treated with L-methionine and *A. armatus* extract, (P) treated with *A. armatus* extract. Values are the means±SEM (n); *p<0.05.

1.2.4. Triglyceride

The results of the determination of TG in F group (0.92±0.36 g/l), P group (0.90 ±0.13g/l) M group (1.19±0.34 g/l), MP group (0.78±0.060 g / l) show that there is a highly and significantly difference between groups (P = 0.008). The Tukey test revealed a significant difference between the groups (F and M) P= 0.008) and the groups (M and PM) P= 0.024 (Figure 22).

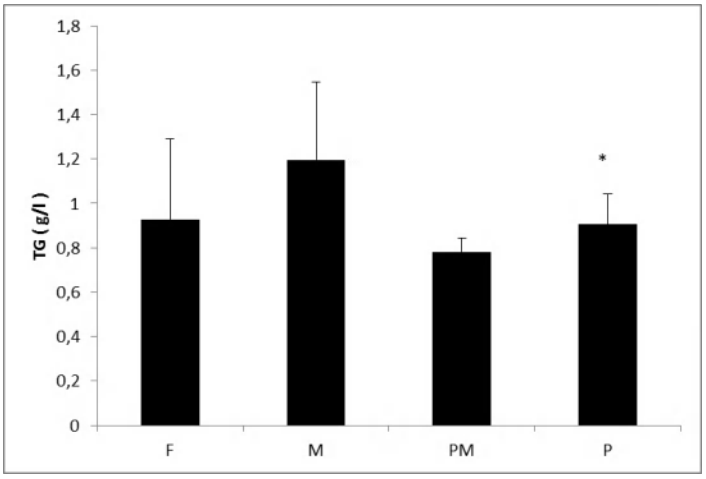

Figure 22. The interaction of L-methionine and *A. armatus* extract on TG in mice, during 21 days of treatment, (F) control group, (M) treated with L-methionine, (PM) treated with L-methionine and *A. armatus* extract, (P) treated with *A. armatus* extract. Values are the means±SEM (n); *p<0.05.

1.3. Antioxidants markers

1.3.1. Glutathione reduced

The concentration of glutathione in the control group F (16.67±2.18 n mol/mg protein), the P group (26.10±7.53 n mol/mg protein), the M group (10.63±7.15 n mol/mg protein) and in the PM group was (17.39±4.08 n mol/mg protein) The values obtained during this study showed a high and significant difference between the groups in terms of mean concentration of GSH (P=0.005). The Tukey test revealed a significant difference between the groups (F and P) P= 0.049) and the groups (P and M) P= 0.002 (Figure 23).

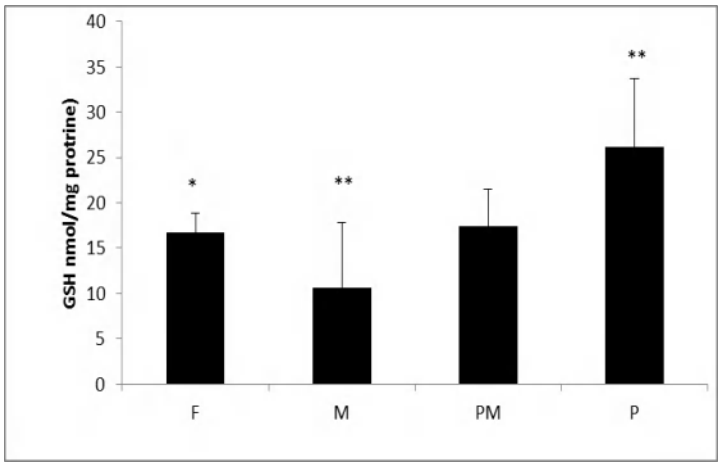

Figure 23. The interaction of L-methionine and *A. armatus* extract on glutathione reduced values (GSH) in mice, during 21 days of treatment, (F) control group, (M) treated with L-methionine, (PM) treated with L-methionine and *A. armatus* extract, (P) treated with *A. armatus* extract. Valuesare the means±SEM (n); (*p<0.05, **p<0.01).

1.3.2. Catalase activity

The concentration of Catalase in the control group F (36.94±18.55 m mol/mg of protein), the P group (53.34±11.51 m mol/mg protein), the M group (21.74±19.86 m mol/mg protein) and in the PM group was (42.28±19.93 m mol/mg protein) show that there is a significantly difference between groups (P=0.042). The Tukey test revealed a significant difference between the groups (P and M) (P=0.028) (Figure 24).

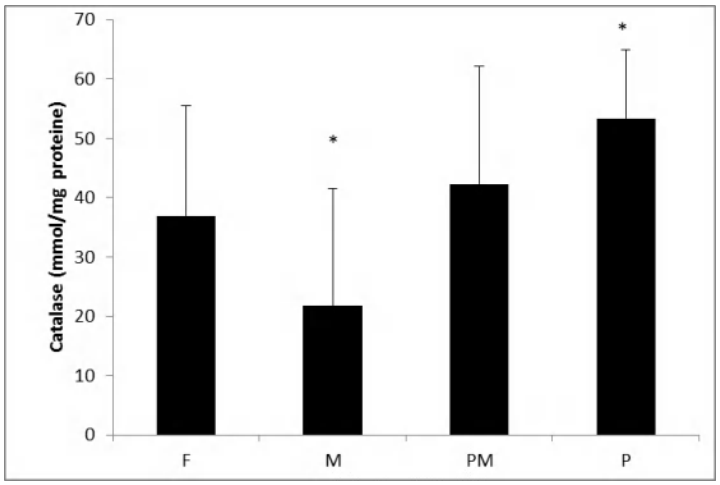

Figure 24. The interaction of L-methionine and *A. armatus* extract on catalase activity (CAT) in mice, during 21 days of treatment, (F) control group, (M) treated with L-methionine, (PM) treated with L-methionine and *A. armatus* extract, (P) treated with *A. armatus* extract. Values are the means±SEM (n); (*p<0.05).

1.4. Histological investigation

1.4.1. Heart

For group (M) which received L-methionine (400mg/kg/day), light microscopy of the heart showed lysis and necrosis in the muscle fiber structure and mononuclear cell infiltration between the muscle cells (Figure 25-C, 25-D). This is not the case for group (F) which presented a normal tissue structure (Figure 25-A, 25-B). Same results were also observed in PM and P groups (Figure 25-E, 25-F).

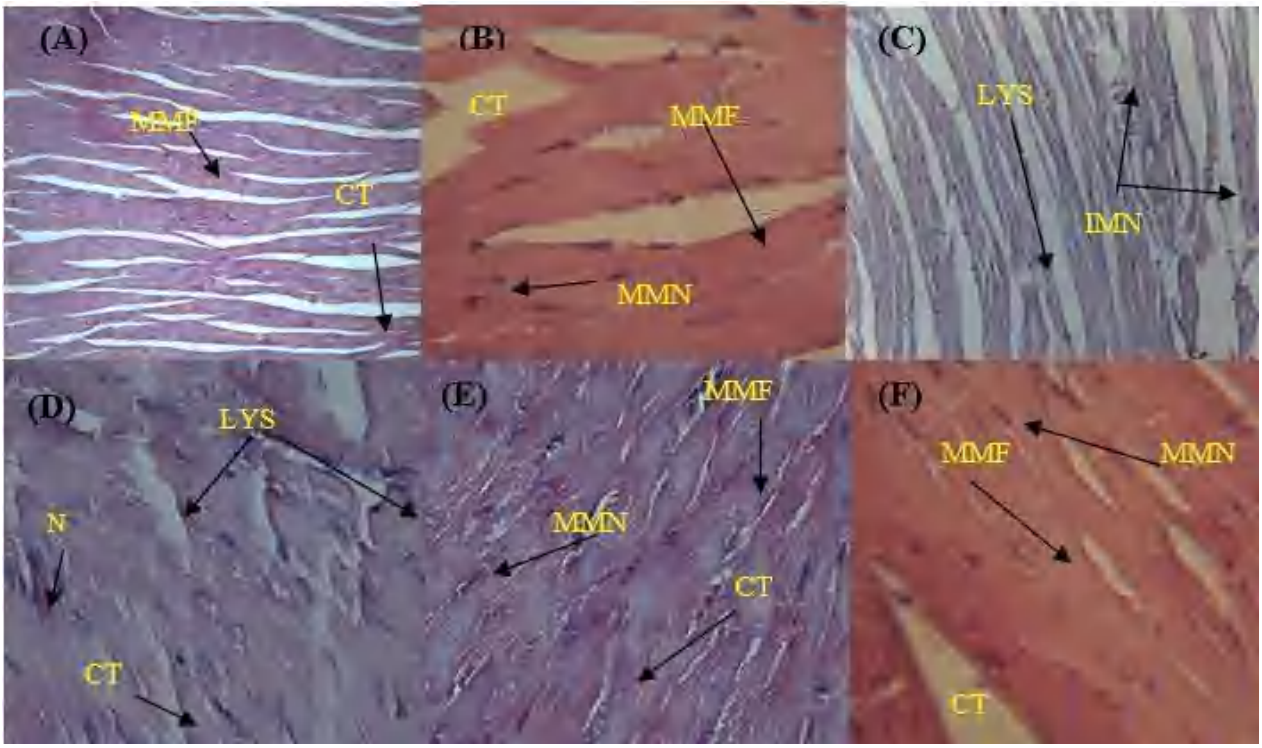

Figure 25. Histological section of the heart, during 21 days of treatment, (A and B) Control group, (C and D) Treated with L-methionine, (E) Treated with L-methionine and *A. armatus* extract, (F) Treated with *A. armatus* extract. Application hematoxylin-eosin staining, (A, C) x100, (B, D, E, F) x400. MMN: Muscular myocard nuclei, MMF: Muscular myocardfibers, CT: Connective tissue. N: Necrosis, LYS: Lysis, IMN: Infiltration of mononuclear.

1.4.2. Aorta

Microscopic observation of aortic intima in the methionine group (M) showed desquamation of endothelial cells, we also observed in the media gaps and formation of oval nuclei (Figure 26- B, 26-C, 26-D). However, in the control group (F).

Histopathological examination showed a normal structure, the aortic section has intact endothelium and spindle shaped nuclei, (Figure 26-A). For the MP and P groups, the observation presents an intact morphology (Figure 26-E, 26-F).

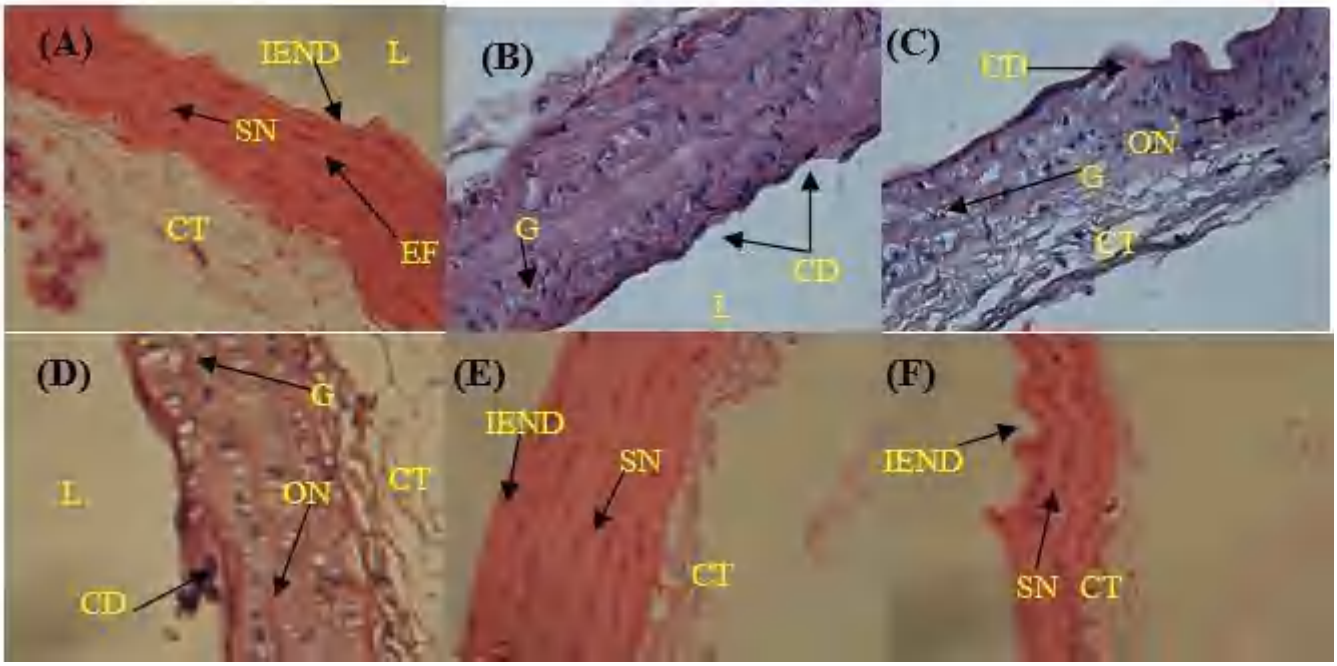

Figure 26. Histological section of the aorta, during 21 days of treatment, (A) control group, (B, C and D) treated with L-methionine, (E) treated with L-methionine and *A. armatus* extract, (F) treated with *A. armatus* extract. Application hematoxylin-eosin staining, (A, F) x100, (B, C, D, E) x400. L: Lumen, IEND: Intact Endothelium, CT: Connective tissue, EF: Elastic Fibers, SN: Spindle nuclei, ON: Oval Nuclei, G: Gaps, CD: Cellular Desquamation.

1.4.3. Liver

Regarding the liver of the control group (F), histopathological examination showed a normal structure (Figure 27-A, 27-B). However, intact liver lesions revealed in group M (mice treated with L-methionine) resulted in severe pathological liver damage marked by necrosis, lysis, change in the shape of hepatocyte nuclei and hepatic steatosis (Figure 27- C, 27-D). For the MP and P groups, the observation presents an intact morphology like that of the control group (Figure 27-E, 27-F).

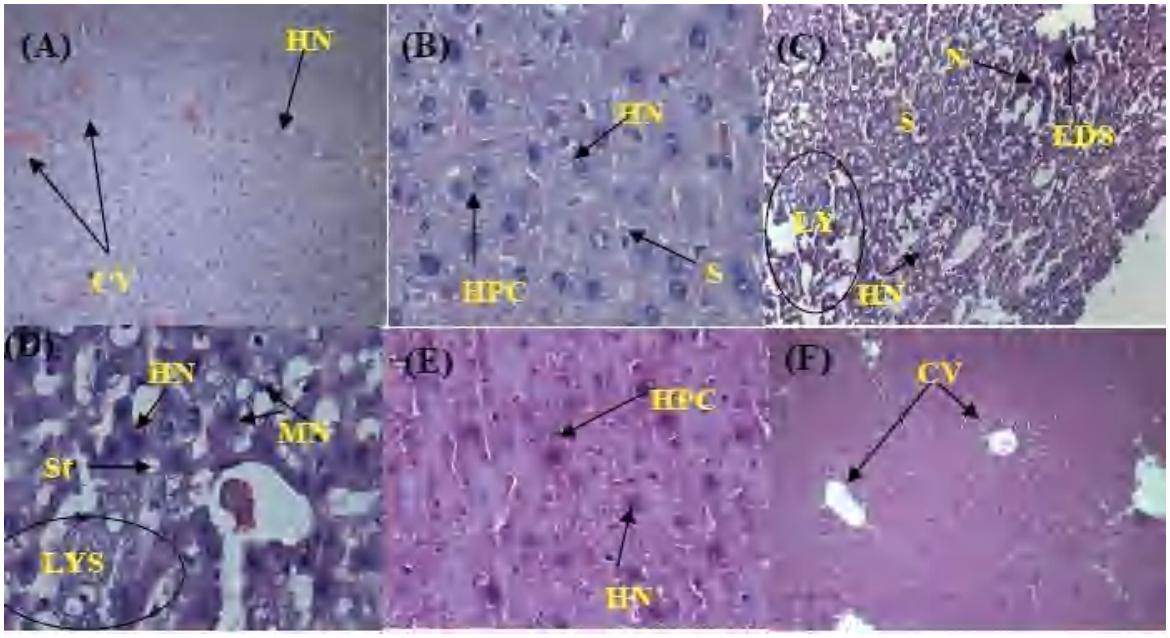

Figure 27. Histological section of the liver, during 21 days of treatment, (A and B) control group, (C and D) treated with L-methionine, (E) treated with L-methionine and *A. armatus* extract, (F) treated with *A. armatus* extract. Application hematoxylin eosin staining, (A, C, F) x100, (B, D, E) x400. HN: Hepatocyte nuclei, HPC: Hepatocyte cell, S: sinusoid, EDS: Endolysis, CV: Central vein, N: Necrosis, ST: Steatose, LYS: Lysis.

2. Tests *in vitro*

2.1. Evaluation of antioxidant activity of n-butanol extract of *A. armatus*

2.1.1. Total antioxidant activity

Table (10) shows the result obtained concerning the total antioxidant activity of the n- BuOH *A.armatus* extract. This extract showed total antioxidant activity but lower than antioxidant standards

2.1.2. Reducing power

The n-butanol extract of *A. armatus* has a low reducing power in comparison with the antioxidant standards: BHA, Ascorbic acid, Trolox (Table 10).

2.1.3. Free radical scavenging activity (DPPH˙)

The n-butanol extract of *A. armatus* has a low Free radical scavenging activity in comparison with the antioxidant standards: BHA, Ascorbic acid, Trolox and a have Free radical scavenging activity higher than the standard antioxidant BHT at 500 µg / mL and equalized at 100 µg / mL (Table 10).

2.1.4. Metal chelating activity

The n-butanol extract of *A. armatus* has a low chelating activity of metals than the antioxidant standard EDTA (Table 10).

Table 10: Total antioxidant activity, reducing power, free radical scavenging and metal chelating activity of n-butanol extract of *A. armatus*, using antioxidant standards as positive controls: EDTA, trolox, BHA, BHT, vitamin C. The values were represented means ± standard deviation (n = 3).

Samples	**In *vitro* Antioxidant Assays**			
	50 µg mL-1	100 µg mL-1	250 µg mL-1	500 µg mL-1
Total antioxidant activity, 695 nm				
n-BuOH *A.armatus* extract	0.141±0.023	0.180±0.015	0.341±0.037	0.593±0.075
BHA	0.228±0.020	0.469±0.024	0.702±0.019	1.699±0.046
BHT	0.199±0.032	0.304±0.032	0.537±0.002	0.875±0.036
Ascorbic acid	0.251±0.030	0.477±0.015	1.097±0.051	2.680±0.052
Reducing power, 700 nm				
n-BuOH *A.armatus* extract	0.086±0.005	0.087±0.015	0.123±0.016	0.143±0.004
BHA	0.265±0.006	0.334±0.014	0.429±0.092	0.465±0.032
BHT	0.269±0.015	0.350±0.011	0.438±0.024	0.564±0.041
Ascorbic acid	0.191±0.048	0.327±0.002	0.366±0.028	0.379±0.031
Trolox	0.213±0.001	0.296±0.006	0.367±0.033	0.386±0.035
Free radical scavenging activity (DPPH˙), %				
n-BuOH *A.armatus* extract	5.80±1.85	24.43±5.76	30.56±4.42	48.26±3.99
BHA	69.48±3.64	77.46±4.61	85.17±5.38	89.29±2.10
BHT	10.02±5.52	25.49±5.89	35.28±8.30	43.38±3.51
Ascorbic acid	74.91±7.43	82.27±6.45	80.05±2.29	84.68±4.81
Trolox	73.83±2.46	86.75±6.18	89.13±4.48	94.24±2.85
Metal chelating activity, %				
n-BuOH *A.armatus* extract	5.53±2.83	6.19±1.82	12.88±2.37	31.08±3.52
EDTA	52.27±6.18	94.01±6.86	97.72±1.47	98.96±0.54

2.2. Evaluation of antiproliferative activity of n-butanol extract of *A. armatus*

2.2.1. Cell proliferation assays

The antiproliferative activities of *A. armatus* BuOH extract and standard were determined relative to the C6 and HeLa cell lines using the BrdU ELISA cell proliferation assay. 5-fluorouracil (5- FU) was used as a positive control (Fig 28-29). The antiproliferative activities of samples and positive control were studied in eight concentrations (5, 10, 20, 30, 40, 50, 75 and 100 µg / mL). The IC50 and IC75 values of the extracts and 5-FU were identified using the ED50 plus v1.0 programs (Table 10).

The figures 28 and 29 indicated that *A. armatus* BuOH extract anhanced the antiproliferative activity in a dose dependant manner. In addition the *A. armatus* BuOH extract was determined to have the higher antiproliferative activity against C6 cells than Hela cells at the concentration 100 µg/mL (Fig 26-27).

However, n-butanol extract of *A. armatus* (IC50: 54.048±0.012 µg/ml) against HeLa cell and (IC50: 79.425±0.030 µg/mL) against C6 cell, has lower antiproliferative activity compared with standard compound at all the concentrations (Table. 10).

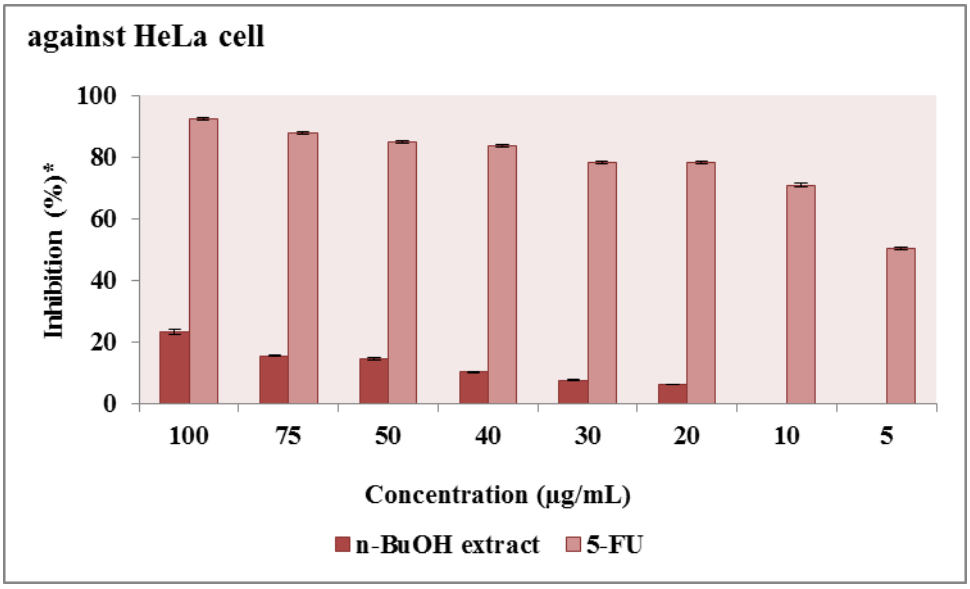

Figure 28. Antiproliferative activity of n-butanol extract of *A. armatus* and 5-FU against the HeLa cell line (3 x 104 cells / well).

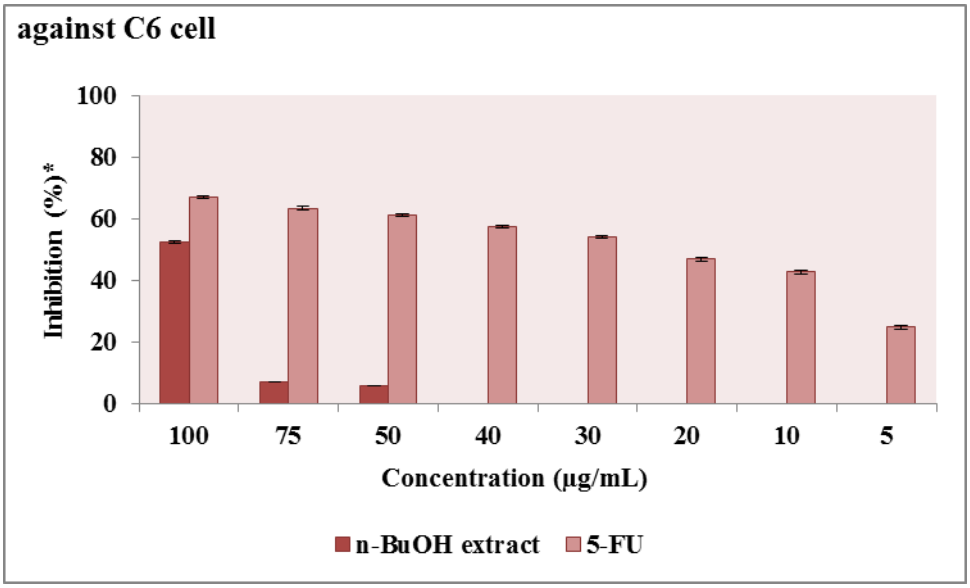

Figure 29. Antiproliferative activity of n-butanol extract of *A. armatus* and 5-FU against the C6 cell line (3 x 104 cells / well).

Table 11: The IC50 and IC75 values of the n-butanol extract of *A. armatus* and 5- fluorouracil, presented by antiproliferative assay against the HeLa and C6 cell lines (3 × 103 cells / mL).

Sample name	HeLa cell		C6 cells	
	IC50 µg/mL	IC75 µg/mL	IC50 µg/mL	IC75 µg/mL
n-butanol extract of *A. armatus*	54.048±0.012	72.964±0.120	79.425±0.030	93.880±0.046
5-FU	>100	13.181±0.020	>100	35.960±0.050

Discussion

Homocysteine is a nonprotein forming, sulfur-containing amino acid, formed during the metabolism of the essential amino acid methionine. Recently, elevated plasma Hcy levels have been impilicated as a risk factor for cardiovascular diseases (**Selhub, 1999**).

Humans and animals produced homocysteine from L- methionine, for this reason we respect this metabolic pathway, we gave the mice L-methionine rather than homocysteine. The main objective of our work is to clarify the effects of a high dose of L- methionine on some biochemical parameters (Hcy, total cholesterol, LDL, HDL, TG, oxidant status) and on the histological structure of certain organs (aorta, heart and liver) in mice and to explore the therapeutic effect of the (EtOAc) extract of A. armatus (100mg/kg) on abnormalities caused by high dose of L-methionine (400mg/kg) during 21 days of treatment.

Our current results showed that L-methionine enriched diet in mice for 21 days caused a significant increase in Hcy concentration, an increase in the concentrations of Tch, LDL-c and TG, decrease in HDL-c, GSH and CAT, these results are associated with the appearance structural alterations of organs aorta, heart and liver.

In the present study, consumption of high L- methionine diet (400mg/kg) during 21 days of the experiment, was sufficient to induce HHcy in mice notable by a highly significant increase of plasma Hcy compared to the control group.

Our results are in agreement with the previous experimental study of (**Boyacioglu *et al.*, 2014; Derouiche *et al.*, 2014; Aklil *et al.*, 2017**), which have shown that consumption of high L-methionine diet induced a significant increase of plasma Hcy.

Hcy levels can markedly fluctuate among different populations due to their dietary habits, depending on the content of dietary methionine, commonly found in poultry diet, and choline. Approximately 50-80 % of generated Hcy, is remethylated to methionine. In humans, the relation between methionine intake and HHcy also depends on vitamin status (folate, vitamins B6 and B12) and the supply of other amino acids (**Koklesova *et al.*, 2021**).

In vivo analysis revealed that a high-Met diet can induce HHcy and can affect epigenetic processes, mainly increased global methylation (5-mC) and DNA methyltransferase-1 (DNMT1) expression. Further, HHcy is associated with increased methylation of CβS promoter in bone marrow-derived endothelial progenitor cells (**Behera *et al.*, 2019**).

This potential effect of high L-methionine diet could possibly be due to up-regulation in the enzymes that metabolize Hcy, for example cystathione β-synthase. It is clear that tissue concentration of Hcy

is maintained at low levels by regulating production and efficient removal of this thiol (**Stipanuk, 2004**).

Derouiche *et al*., (2014) reported that HHcy is a consequence of genetic defects of some enzymes, nutritional deficiencies, methionine rich diet, related of some diseases and some drugs.

HHcy leads to diverse clinical manifestations; the pathogenicity of homocysteine is believed to be due to its ability to produce oxidative stress (**Noll *et al*., 2011**), HHcy remains a major risk factor leading to endothelial cell dysfunction and induces apoptotic cell death through reactive oxygen species (ROS) production in endothelial and smooth muscle cells. HHcy also causes accumulation of damaged proteins resulting in modification and alterations of their function (**Zhang *et al*., 2001**).

High plasma Hcy concentrations may increase in different pathophysiological conditions (renal failure, rheumatoid arthritis and B-vitamins deficiencies etc.), which is considered as a risk factor for cardiovascular diseases. The elevation of plasma Hcy levels may contribute to ischemic changes and oxidative stress. HHcy produces changes instructure and function of blood vessels and oxidative stress appears to play a major role in mediating these changes (**Boyacioglu *et al*., 2014**). It has been suggested that HHcy is associated with ROS formation, such as superoxide anion, hydrogen peroxide and hydroxyl radicals which are normally eliminated by antioxidants enzymes (**Faraci and Lentz, 2004**).

Hcy has also the ability to inhibit the expression of antioxidant enzymes such as glutathione peroxidase (GSH-Px) which might destroy the toxic effects of reactive oxygen species (ROS). As a result, oxidative stress which is induced by homocysteine results in the damage of vascular endothelial cell (**Celik *et a*l., 2016**).

HHcy is an important risk factor for cardiovascular diseases (**Boyacioglu *et al*., 2014**), and is thought to produce endogenous oxidative stress and causes many cellular damages (**Derouiche *et al.*, 2014**). An important role in development of hyperhomocysteinemia is also played by deficiency of folic acid, vitamins B6 and B12 (**Domagala *et al.*, 1997**),

HHcy is often related to age and race physiological particularities as well as individual genetic, epigenetic, nutritional, and latrogenic (drugs) risk factors, among others. At the same time, the leading cause of HHcy is related to an insufficient amount and/or dysfunction of enzymes and cofactors (water-soluble vitamins B2, B6, B9, and B12) associated with the metabolism of Hcy, especially in the elderly population. HHcy can be related to increased Hcy production by transmethylation, decreased Hcy removal by transsulfuration or remethylation, or a decrease in the

Hcy excretion (**Koklesova *et al.*, 2021**), however, may be prevented by polyphenols, potent antioxidant compounds with anti atherogenic properties (Noll et al., 2011). The use of vitamins to modulate homocysteine metabolism substantially lowers the risk by reducing plasma homocysteine levels (**Sim *et al.*, 2016**).

Further, we found that the treatment with the (EtOAc) extract of *A. armatus* (100mg/kg) was effective in protection against HHcy in mice fed a diet rich in L-methionine by decreasing plasma Hcy levels, indicating that this medicinal plant has the potential to reduce t-Hcy levels in mice.

Our results are in agreement with the previous experimental study (**Aklil *et al.*, 2017; El-Saleh *et al.*, 2004; Haddadi *et al.*, 2017**). They reported that HHcy was induced by the high dose of L-methionine in mice and rats and confirmed the significant protective role of *Argania spinosa* extract, *Thymoquinone* and *Nigella sativa* oil and lyophilized prune extract against situational HHcy.

In vitamin therapy, it could be also combined to an antioxidant treatment obtained from natural phytochemicals, a source for natural antioxidants to protect against the homocysteine mediated free oxygen radicals damages (**Benmebarek *et al.*, 2013**).

Recent studies have proven that antioxidants can lower Hcy levels very effectively (**El-Saleh *et al.*, 2004; Çelik *et al.*, 2017**). The study of **Labed *et al.*, (2016**) show that the ethyl acetate extract of *A. armatus* contain a flavonoid and this extract exhibited the highest antioxidant activity in DPPH, ABTS and CUPRAC assays. Phenolic compounds as well as flavonoids are well-known as antioxidant, which are responsible for their health benefits, curing and preventing many diseases (**Tungmunnithum *et al.*, 2018**). The protective effect of the (EtOAc) extract of *A. armatus* against situational HHcy is probably due to its high content of flavonoid compound known as powerful antioxidant.

Furthermore, we detected an increase in the concentrations of Tch, LDL-c and TG, as well as a decrease in HDL-c following the oral administration of L-methionine, our result demonstrated a positive correlation between Hcy and the lipid profile (Tch, LDL-c and TG) as well as a negative correlation with HDL-c. This is in agreement with the previous experimental study by (**Obeid *et al.*, 2009; Momin *et al.*, 2017; Shaker *et al.*, 2013**).

An association between hyperlipidemia and HHcy has been suggested. The present study showed that HHcy was independently associated with hypertriglyceridemia and low levels of HDL-C, which provides evidence that Hcy levels might affect HDL-C and TG metabolism **(Momin *et al.*, 2017)**.

Hyperlipidemia occupies a critical position in the development of cardiovascular diseases, non-alcoholic fatty liver diseases (NAFLD) and other metabolic diseases. Changes in dietary habit make hyperlipidemia an issue of public concern, which is characterized by abnormally elevated serum levels of cholesterol and/or triglyceride with or without low levels of HDL cholesterol (**Deng *et al*., 2018**).

However, results from (**Liao *et al*., 2006**) indicated that HHcy inhibits reverse cholesterol transport by reducing circulating HDL-c. This is done through inhibiting apoA-I protein synthesis and enhancing HDL-c clearance. Also the study of (**Obeid *et al*., 2009)** demonstrate that the effect of Hcy on HDL-cholesterol is probably related to the inhibition of enzymes or molecules participating in the HDL-particle assembly.

Many studies suggest that Hcy-induced HDL-C and apoA-I inhibition represent a novel mechanism by which Hcy induces atherosclerotic (**Liao *et al*., 2007**).

In apoE-null mice, deletion of the gene for cystathionine -synthase (CβS), which converts homocysteine (Hcy) to cystathionine, leads to severe HHcy and increased aortic lesions that are associated with increased plasma total cholesterol (Tch) and decreased high-density lipoprotein cholesterol (HDL) (**Wang *et al*., 2003**).

The increase in LDL seems to be linked to their peroxidation by free radicals generated by HHcy because this peroxidation inhibits the recognition of oxidized LDL by their native receptors (**Laporte, 2000**). The work of **Werstuck *et al*., (2001**) proved that accumulation of hepatic cholesterol and triglycerides in HHcy is associated to an increase in the biosynthesis and absorption rather than a reduction in the hepatic export of VLDL.

Homocysteine is toxic to vascular endothelium, it promotes thrombosis and potentiates the oxidation of low density lipoprotein (LDL) *in vitro*. It has been suggested lately that oxidation of LDL may be one of the main factors involved in initial development of atherosclerotic lesions (**Domagala *et al*., 1997**).

In addition, the administration of the (EtOAc) extract of *A. armatus* with L-methionine caused a decrease in lipid parameters (Tch, LDL-c and TG), showing the beneficial effect of this plant in the treatment of the hyperlipidemia. This protection related to the decrease level of Hcy and therefore the suppression of their cytotoxic effects on different organs.

Due to the severe consequences of hyperlipidemia, the prevention and control of lipid metabolic disorder is imperative. Although many conventional pharmaceuticals are currently available for the

therapy of hyperlipidemia or some new target therapeutic drugs are under scrutiny in the clinical research, they may have significant adverse effects on health, such as myositis, myopathy and hepatic enzyme abnormalities (**Mahamuni *et al*., 2012**).

Investigations have revealed that polyphenols play a key role to prevent various diseases, like hypercholesterolemia, hyperglycemia, hyperlipidemia, and cancer insurgence (**Abbas *et al*., 2017**).

The findings of the present work indicate that a MeOH extract of *P. Auidiatu* stems and it flavonoids, may be useful for the treatment of hyperlipidemic disease (**Choi *et al*., 1991**).

The study of **Seo *et al*., (2010**) proved that high intake of antioxidants appeared to be protective factor against atherosclerosis, possibly exerting a pro-oxidative effect on LDL when combined with high levels of Hcy and LDL. Recent evidence suggests that some polyphenols in their purified form have beneficial effects on dyslipidemia in humans and animal models (**Mulvihill and Huff, 2010**).

The administration of the (EtOAc) extract of *A. armatus* with L-methionine caused a decrease in lipid parameterse showing the beneficial effect of this plant in the treatment of the hyperlipidemia, these corrections related to the antioxidants components particularly flavonoid of *A. armatus* extract.

The antioxidant activity of polyphenolics is principally defined by the presence of orthodihydroxy substituents, which stabilize radicals and chelate metals. The antioxidant effect of phenolic acids and their esters depends on the number of hydroxyl groups in the molecule. This antioxidant compound protects against LDL-oxidation (**Chen *et al*., 2004**).

Furthermore, our data showed a decrease in GSH and CAT activities as a biochemical marker of oxidative stress in mice given a high dose of L-methionine. Our findings are supported by the study of (**Aklil *et al*., 2017**) who reported a decrease in GSH and CAT levels of mice treated with 400 mg/kg of L-methionine for 21 days.

To cope with the damaging actions of ROS, organisms have evolved a sophisticated ROS defense system (RDS), consisting of low-molecular-weight antioxidants, such as glutathione, ascorbic acid, tocopherol, and uric acid, and specialized ROS-detoxifying enzymes, such as superoxide dismutases (SODs), catalase (CAT), glutathione peroxidases (GPxs), and various thio-, peroxi-, an glutaredoxins (**Andreyev *et al*., 2005; Rhee *et al*., 2005**).

Failure of RDS to cope with the intracellular ROS production results in oxidative stress, which contributes to the damage and death of cells. Therefore, measuring the activity of RDS enzymes is a valuable diagnostic tool to determine the role of the oxidative stress in the pathology of a particular disease **(Cristofol, 2007**).

Hcy reduced enzymatic antioxidant potential in tissues. Moreover, oxygen radicals may play an important role in this specific HHcy model (**Boyacioglu *et al*., 2014**), long-term methionine treatment promotes oxidative stress as it decreases non-enzymatic antioxidant defenses, increases lipid peroxidation and carbonyl content, alters activity of antioxidant enzymes, and changes in serum biochemical parameters (**Stefanello *et al*., 2009 and Matté *et al*., 2009**).

Da Cunha *et al*. (2011) have shown that Hcy significantly reduced GSH content and G6PD activity after chronic Hcy administration. G6PD is the key regulatory enzyme of the pentose phosphate pathway, which could promote impairment in the production of NADPH and a disruption in the cellular redox balance. This is probably in line with the observed inhibition of GPx activity at 12 h, since the activity of this enzyme depends on the regeneration of reduced glutathione by glutathione reductase, which in turn relies on NADPH that is dependent on a normal G6PD activity (**Hashida, 2002**) .

In addition to increasing H2O2 generation, Hcy decreases the cell's ability to detoxify H2O2 by impairing intracellular antioxidant enzymes, specifically the intracellular isoform of glutathione peroxidase (GPx) (**Upchurch *et al*., 1997**).

On the other hand, the decrease in CAT and GPx activities may be explained by the fact that antioxidant enzymes are inhibited by specific ROS, which are probably formed from Hcy. We believe that this imbalance between antioxidant enzymes probably alters ROS elimination (**Fridorich, 1986; Vessey and Lee, 1993)** , which are probably formed from Hcy (**Heinecke, 1987**).

Catalase, an antioxidant enzyme responsible for the hydrogen peroxide degradation, is protective in many diseases. Catalase inhibition by homocysteine may increase the levels of hydrogen peroxide and play a role in the pathology of disease. (**Nathaniel, 2008**). The inhibition of catalase may be the mechanism for homocysteine enhancement of inhibitor amyloid-ß (**Milton, 1999**).

Further, administration of the (EtOAc) extract of *A. armatus* in combination with methionine rich diet elevated plasma GSH and CAT levels, The (EtOAc) extract of *A. armatus* has been confirmed to contain flavonoid wich indicated the highest antioxidant activity (**Labed *et al*., 2016**), the antioxidative properties of flavonoids are favoured by a high degree of OH substitution. On the other hand, inhibition of enzymatic functions other than oxidases, e.g., inhibition of lipoxygenase and thus prevention of the formation of leukotrienes, may also participate in the cell and tissue protective properties of flavonoids. (**De Groot and Ruin, 1998**).

Our results are in agreement with previous experimental research by (**Aklil *et al*., 2017; El-Saleh *et al*., 2004; Çelik *et al*., 2017; Meng *et al*., 2013**), they demonstrated that the *Argania spinosa* extract, *thymoquinone* or black seed oil and quercetin treatment could have a preventive effect against oxidative stress produced by homocysteinemia in rats.

One of the possible mechanisms of the protective effect of quercetin could be associated to its antioxidant properties, which rise antioxidant enzyme activities like SOD, CAT and GSH level, non-enzymatic antioxidant, in plasma (**Meng *et al*., 2013**).

Flavonoids are also responsible for the stimulation of antioxidant enzymes. Its ability to trigger the generation of antioxidant enzymes in human body. (**Soto *et al*., 2003**).

A.armatus extract is rich en flavonoids. These compounds have been found to modulate expression and activity of catalase and eNOS in several tissues, increases catalase activity in guinea pig cardiac tissue (**Floreani *et al*., 2003**).

Cardiovascular diseases and liver damage are major public health problems. HHcy has been associated with cardiovascular disease, and defects in methyl group metabolism are among key molecular events thought to play a role in liver injury (**Selicharová *et al*., 2013**). Oxygen free radicals, caused by HHcy, affected not only heart tissue, but also liver and renal tissues (**Boyacioglua *et al*., 2014**).

Histopathological examinations in hyperhomocysteinemic mice, showed an appearance of structural alterations on the aorta, heart and liver tissue damages. This was observed through lysis and necrosis in the muscle fiber structure, and mononuclear cell infiltration between the muscle cells in the heart tissue, the aortic intima showed desquamation of endothelial cells, we also remarked in the media lysis, gaps, formation of foam cells and oval nuclei, our results are in agreement with (**Aklil *et al*., 2017; Benmebarek *et al*., 2013**) which reported that the oral administration of L-methionine exerted an angiotoxic activity on the aorta and a toxic effect on the heart.

However, liver lesions were observed in mice treated with L-methionine resulted in severe pathological liver damage marked by necrosis, change in the shape of hepatocyte nuclei and hepatic steatosis. Our results agree with the study of (**Benmebarek *et al*., 2013 and Taravati *et al*., 2013**), which reported that an excess of deity methionine causes a toxic effect on liver and heart tissues.

A moderate elevation of plasma homocysteine is a risk factor for atherosclerosis and arterial and veinous thrombosis responsible for coronary heart disease and ischemic stroke incidence and

cardiovascular disease mortality (**Bhandari *et al.*, 2011**).

HHcy acts via oxidative stress to promote myocardial fibrosis and dysfunction (**Joseph *et al.*, 2008**). Increased oxidant stress appears to play a pathophysiological role in the deleterious endothelial predispose affected vessels to the subsequent development of atherosclerosis predispose affected vessels to the subsequent development of atherosclerosis effects of homocysteine (**Kanani *et al.*, 1999**). An association between the presence of abdominal aortic aneurysm and high homocysteine plasma levels has been mentioned, homocysteine plays an important role in development of aortic dissection (**Takagi *et al.*, 2005**), the results of (**Lamda *et al.*, 2014**) show that elevated plasma homocysteine increase cholesterol synthesis, exerts an angiotoxic action direct to aorta through loss of endothelium, degeneration partly with dissolution of media cells.

Hcy induced oxidative damages both in heart and aorta by increasing lipid peroxidation and oxidized proteins since MDA, a biomarker of lipid peroxidation, a parameter of protein damage were higher. This condition could be related to increasing ROS generated in part by Hcy autoxidation and also may be mainly related to mitochondria that generated more ROS in response to oxidative stress as previously reported (**Faraci *et al.*, 2004**).

Homocysteine has also been shown to in crease DNA synthesis in vascular smooth muscle cells consistent with early arteriosclerotic lesions and to induce these cells to proliferate while impeding the regeneration of endothelial cells (**Tsai *et al.*, 1994**).

Experimental studies demonstrated that HHcy induces endothelial dysfunction (**Bellamy *et al.*, 1998)**, collagen synthesis (**Majors et *al.*, 2002**), and deterioration of the elastic material of the arterial wall through increased activation of matrix metalloproteinases (**Vizzardi *et al.*, 2009**). Acute methionine-induced HHcy has been found to decrease NO synthesis and release, through an inhibition of NO-synthase activity (**Romerio *et al.*, 2004**). Thus, it is possible that reduced NO bioavailability is responsible for the impairment of aortic distensibility observed in our participants after the methionine load (**Eleftheriadou *et al.*, 2013**).

The effects of homocysteine on vascular hemostatic properties have included decreased thrombomodulin cell surface expression and inhibition of protein C activation, thus probably contributing to development of thrombosis (**Lentz *et al.*, 1991**).

Histological analysis indicated the presence of inflammatory infiltrate, fibrosis and reduced content of glycogen/glycoprotein in liver tissue sections from hyperhomocysteinemic rats (**Matte´*et al.*, 2009**). HHcy results from a hepatic metabolism dysfunction and is characterized by a high plasma

homocysteine level, which lead to hepatic lesions and abnormal lipid metabolism (**Latour *et al.*, 2015**). The study of (**Noll *et al.*, 2011**) found that HHcy leads to a decrease in hepatic Nqo1 gene expression and activity with a reversal opposite effect of polyphenol extract (PE) supplementation.

Since the liver has an important role in the Met metabolism (**Finkelstein, 1990**), it has been proposed that elevated levels of this amino acid can be highly hepatotoxic (**Hardwick *et al.*, 1970**). In this context, some data from the literature showed that disturbances of the Met cycle might result in hepatic damage, such as liver cirrhosis (**Avila *et al.*, 2005**).

While the administration of the (EtOAc) extract of *A. armatus* with L-methionine caused an improvement in histological changes, and corrected the structural abnormalities observed in the aorta, heart, and liver tissues. The work of (**Aklil *et al.*, 2017; Benmebarek *et al.*, 2013**) proved that, HHcy induced by the high methionine diet, could damage the aorta, heart and liver tissue, and the treatment of these animals with *Argania spinosa* powdered seeds and *Stachys mialhesi* extract respectively corrected these alterations. The results of **(Yalçınkaya *et al.*, 2009**) indicated that taurine has protective effects on HHcy induced toxicity by decreasing oxidative and nitrosative stresses, apoptosis, and necrosis in the liver.

Possible protective effects against heart disease may be due to the ability of some polyphenols to prevent the oxidation of LDL to an atherogenic form although anti-platelet aggregation activity and vasodilatory properties are also reported (**Duthie *et al* .,2000**)

The (EtOAc) extract of *A. armatus* has been confirmed to contain flavonoid wich indicated the highest antioxidant activity (**Labed *et al.*, 2016**), the antioxidative characteristic of flavonoids are favored by a high degree of OH substitution. Furthermore, inhibition of enzymatic functions other than oxidases, like inhibition of lipoxygenase and thus protection of the formation of leukotrienes, may also participate in the cell and tissue preventive properties of flavonoids (**De Groot and Ruin, 1998**).

The results of evaluation antioxidant activity of n-BuOH *A. armatus* extract by four methods: Total antioxidant by phosphomolybdenum assay, assay of reducing power, free radical scavenging assay, and metal chelating assay showed a low activity. This result can be remarkable compared to the study by (**Labed *et al.*, 2016**) which shows that the antioxidant activity of the AcOEt fraction higher than that of the butanol fraction of *A. armatus* extract, this is probably due to the richness of the AcOEt fraction in aglycone flavonoids like isolated isorhamnetine, in fact it has the first two criteria that influence the quality of the antioxidant activity, as for the butanolic fraction, it contains 3-position

glycosylated flavonoids, significantly reducing antioxidant activity.

Cancer is a major public health burden in both developed and developing countries. Anticancer activity is the effect of natural and synthetic or biological and chemical agents to reverse, suppress or prevent carcinogenic progression. Several synthetic agents are used to cure the disease but they have their toxicity and hence the research is going on to investigate the plant derived chemotherapeutic agents (**Shaikh *et al.*, 2016**). Antiproliferative activities are widely studied for medicinal plants and other common sources (**Demirtas *et al.*, 2009)**.

The anticancer properties of n-BuOH *A. armatus* extracts against HeLa and C6 cells were tested using the BrdU ELISA cell proliferation assay and compared to the positive control of 5-florourasil (5-FU). The extract exhibited various anticancer effects at different concentrations, the antiproliferative activities of n-BuOH *A. armatus* was shown to increase of the activities depending to dose increasing against HeLa and C6 cells. In addition to, the n-BuOH *A. armatus* was determined to have the higher antiproliferative activities against C6 cells than against HeLa cells at the concentration 100 µg/mL. However, n-BuOH *A. armatus* extract (IC50: 54.048±0.012 µg/mL against HeLa cell and IC50: 79.425±0.030 µg/mL against C6 cell) has lower antiproliferative activity compared with standard compound at all the concentrations. The potency of inhibitions (at 100) against HeLa and C6 cells were: 5-FU> n-BuOH extract.

The phytochemical study of n-BuOH *A. armatus* gave four flavonoids (**Labed *et al.*, 2016**), flavonoids are plant bioactive compounds of great interest in nutrition and pharmacology, due to their remarkable properties as antioxidant, anti-inflammatory, antibacterial, antifungal and antitumor drugs (**Fernandez *et al.*, 2021**), Studies in vitro and in vivo have shown that some flavonoids modulate the metabolism and disposition of carcinogens and can contribute to cancer prevention (**Senderowicz, 2001; Carroll *et al.*, 1998**). Flavonoids have been shown to induce apoptosis in some cancer cell lines, while sparing normal cells, flavonoids are particularly effective at inhibiting xanthine oxidase, and therefore inhibit tumor cell proliferation (**Ren *et al.*, 2003**).

Flavonoids have antiproliferative effects and induce apoptosis in different cancer cell lines. As free radical scavengers, flavonoids inhibit invasion and metastasis **(Di Carlo *et al.*, 1999; Kuntz *et al.*, 1999**). **Nijveldt *et al.* (2001**) reported that flavonoid compounds were cytotoxic for cancer but not for normal cells. Flavones also regulate macrophage function in cancer cell elimination and are potential inhibitors of cell proliferation (e.g., apigenin and luteolin). An inverse relationship exists between flavonoids in the diet and the occurrence of lung cancer (**Kro´l *et al.*, 1995; Nijveldt *et al.*, 2001**).

Conclusion and Perspectives

In this research work, we have demonstrated the effect of hyperhomocysteinemia on cardiovascular disease, and explored the possibility of influencing hyperhomocysteinemia by administration of high dose of L-methionine (400mg/kg) during 21 days in an *in vivo* animal. Therefore evaluate the protective and preventive effect of the AcOEt *A. armatus* extract against the metabolic and structural disorders induced in L-methionine treated mice.

On the other hand, evaluation of antioxidant activity and antiproliferative activity of n-BuOH *A. armatus* extract were carried *in vitro*.

Consumption of high L- methionine diet (400mg/kg) during 21 days, resulted in:

- A significant increase in plasma Hcy. Furthermore, we detected an increase in lipid parameters concentrations, and a decrease in HDL-c, glutathione reduced (GSH) and catalase (CAT) activities.
- Histopathological examinations in hyperhomocysteinemic mice, showed;
- An appearance of structural alterations on the aorta, heart and liver tissues damages, This was observed through lysis and necrosis in the muscle fiber structure, and mononuclear cell infiltration between the muscle cells in the heart tissue, the aortic intima showed desquamation of endothelial cells;
- We also remarked in the media lysis, gaps, formation of foam cells and oval nuclei.
- However, intact liver lesions revealed in mice treated with L-methionine resulted in severe pathological liver damage marked by necrosis, change in the shape of hepatocyte nuclei and hepatic steatosis.

While the administration of the AcOEt *A. armatus* extract (100mg/Kg) with L-methionine (400mg/Kg) caused: a decrease in Hcy concentration and lipid parameters, an increase in GSH and CAT activities, and an improvement in histological changes.

However the results of evaluation of antioxidant activity *in vitro* of the n-BuOH *A. armatus* extract by four methods: Total antioxidant by phosphomolybdenum assay, assay of reducing power, free radical scavenging assay, and metal chelating assay showed a low activity.

While the anticancer properties of n-BuOH *A. armatus* extracts against HeLa and C6 cells were tested using the BrdU ELISA cell proliferation assay and compared to the positive control of 5-florourasil (5-FU). The extract exhibited anticancer effects at different concentrations, the

antiproliferative activities of n-BuOH *A. armatus* extract was shown to increase of the activities depending to dose increasing against HeLa and C6 cells. In addition to, the n-BuOH *A. armatus* extract was determined to have the higher antiproliferative activities against C6 cells than against HeLa cells at the concentration 100 µg/mL.

The phytochemical study of n-BuOH *A. armatus* extract gave four flavonoids, this preventive protection of *A. armatus* extract, might be related to its content of many antioxidants and a flavonoid compounds known as powerful antioxidant.

The results obtained in this study show that the plant *A. armatus* can be considered as natural source in the prevention against cardiovascular and cancer diseases.

Our future research and views can assess a wide range of subjects based on the results of this study, including:

- Determination of antioxidant enzyme superoxide dismutase and glutathione-s- Transferase.
- Study the gene expression of antioxidant enzymes.
- Determination of pro-inflammatory cytokines in mice administered with high dose of L-methionine and treated with *A. armatus.*
- Isolate the specific antibacterial principles in *A. armatus.*

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الملخص بالعربية

المقدمة:

تم اكتشاف الهوموسيستين (Hcy) في عام 1932، من طرف العالمين Butz وDu Vigneaud عندما قاموا بتسخين الميثيونين في حمض الكبريتيك وحصلوا على مادة ذات خصائص مشابهة للسيستين، والتي أطلقوا عليها إسم "الهوموسيستيين" ، لأنها كانت متجانسة مع السيستيين (Tsiami and Obersby, 2017).

اقترح العالم McCully لأول مرة فرضية مرض تصلب الشرايين وارتباطه بالهوموسيستين في عام 1969، بعد ملاحظته تجلط شرايين القلب التاجية و شرايين الدماغ و الأطراف لدى الأطفال الذين يعانون من تراكم الهوموسيستيين في البول أو ما يسمى ببيلة الهوموسيستيين، أثناء تشريح الجثث (Kaul *et al*., 2006).

الهوموسيستيين هو حمض أميني كبريتي، و هو ناتج عن استقلاب الميثيونين، والذي يتطلب حمض الفوليك، فيتامين ب 12 و فيتامين ب 6 (Selhub, 1999).

تتمثل الوظيفة الرئيسية للهوموسيستيين العمل كوسيط كيميائي حيوي في استقلاب الميثيونين والتخليق الحيوي للسيستيين ، والذي يلعب أدوارًا مهمة في جسم الإنسان (Mishra, 2016).

ارتفاع أو فرط الهوموسستئين في الدم (HHcy)، هو اضطراب يسبب مخاطر واسعة النطاق على صحة الإنسان (Zhang *et al*., 2005)، يتميز بزيادة مستوى الهوموسستئين في البلازما و هو يرتبط بأمراض القلب والأوعية الدموية منها تصلب الشرايين والإجهاد التاكسدي (Mendes *et al*., 2014)، بالإضافة إلى الإصابات الكبدية والإستقلاب غير الطبيعي للدهون (Latour *et al*., 2015).

يسبب أيضا ارتفاع الهوموسيستيين زيادة في توليد الجذور الحرة منها H2O2، وتقليل نشاط مضادات الأكسدة الإنزيمية مثل الجلوتاثيون بيروكسيداز والكاتلاز ، وبالتالي تعزيز زيادة الإجهاد التأكسدي (Rodrigo *et al*., 2003).

أظهرت العديد من الدراسات أن العديد من مضادات الأكسدة الفينولية والمستخلصات النباتية مثل القهوة، الكاتشين، المركبات الفينولية وحمض الكلوروجينيك تقلل من مستويات الهوموسيستيين في الدم (Nygard *et al*., 1997; Noll *et al*., 2013; Noll *et a*l., 2011; Kim *et al*., 2012).

منذ القدم كانت النباتات مصدرًا أساسيًا للمركبات الطبية ، وبالتالي فإن العديد من الأدوية الرائجة مشتقة بشكل مباشر أو غير مباشر من النباتات (Veeresham, 2012).

نبات *Astragalus armatus* هو نوع مستوطن في الجزائر و المعروف شعبياً باسم قتاد، و قد أكدت دراسة (Khalfallah *et al*., 2014) أن الأجزاء الهوائية منه تحتوي على عدد من المركبات النشطة بيولوجيًا منها جليكوسيدات الفلافونول وثلاثي الجليكوسيد مثل النرجسين ، نيكوتيفلورين و موريتيانين.

من جهة أخرى تم الحصول على الفلافونويد Isorhamnetin من مستخلص إيثيل أسيتات من نبتة *A. armatus* ، هذا الأخير أشار إلى نشاط كبيرفي التفاعل المضاد للأكسدة في فحوصات DPPH ، ABTS و CUPRAC (Labed *et al*., 2016).

تهدف هذه الدراسة إلى التعرف على التأثير الفعال و الوقائي لمستخلص نبتة *A. armatus* على حالة فرط الهوموسيستيين، النشاط المضاد للأكسدة، و على على النمو والنشاط التكاثري لبعض الخلايا السرطانية، و هذا استنادا للدراسات السابقة التي أثبتت دور و فعالية هذه النبتة في علاج بعض الأمراض.

و من هذا المنطلق تتمحور أهداف هذه الدراسة فيما يلي:

- التحفيز على تكوين حالة فرط الهوموسيستيين في الدم لدى الفئران و ذلك باستخدام جرعات عالية من الميثيونين بمقدار﴿ 400 ملغ/ كلغ﴾ لمدة 21 يوم.
- تقييم تأثير الجرعة العالية من الميثيونين (400 مغ/ كغ) على بعض التحاليل البيوكيميائية و قياس نسبتها في الدم مثل قياس نسبة الهوموسيستيين (Hcy)، الكوليسترول، الدهون الثلاثية (Triglycerides)، البروتينات الدهنية عالية الكثافة(HDL-c)، البروتينات الدهنية منخفضة الكثافة(LDL-c)، بالإضافة إلى مضادات الأكسدة الإنزيمية مثل الجلوثاتيون (Gluthatione) و الكاتالاز Catalase.
- إختبار تأثير هذه الجرعات العالية كذلك على المقاطع النسيجية لكل من القلب، الشريان الأورطي و الكبد لدى الفئران.
- تقييم التأثير الوقائي لكسر أسيتات الإيثيل (AcOEt) من نبتة *A. armatus* على الهوموسيستيين و باقي التحاليل البيوكيميائية السابق ذكرها، عند الفئران المتغذية على المثيونين بمقدار ﴿400 ملغ/ كلغ﴾ لمدة 21 يوم.

- تقييم التأثير الوقائي لكسر أسيتات الإيثيل من نبتة *A. armatus* على المقاطع النسيجية للقلب، الشريان الأورطي والكبد عند الفئران المتغذية على المثيونين بمقدار (400 ملغ/ كلغ) لمدة 21 يوم.
- تقييم النشاط المضاد للأكسدة للكسر البوتانولي (n-BuOH) لنبتة *A. armatus* باستعمال عدة طرق هي على التوالي : الفعل المضاد للأكسدة الكلية، القدرة الإختزالية، القدرة على إقتناص الجذور الحرة و استخلاص المعادن.
- تقييم التأثير الوقائي للكسر البوتانولي (n-BuOH) لنبتة *A. armatus* على النمو والنشاط التكاثري لبعض الخلايا السرطانية مثل خلايا HeLa وخلايا C6.

الطرق و الوسائل المستعملة

أولا : تقييم التأثير الوقائي لكسر خلات الإيثيل (AcOEt) من نبتة *A.armatus* على الهوموسيستين البلازمي ،الدهون ، مضادات الأكسدة الإنزيمية، وأنسجة الشريان الأورطي، القلب والكبد في حالة فرط الهوموسيستين الناجم عن التغذية بالميثيونين في الفئران.

1 . المستخلصات النباتية:

خلال هذه الدراسة تم إستخدام نبتة طبية جزائرية تعرف باسم قتاد و هي نبتة *Astragalus armatus*، تم جمع الأجزاء الهوائية من نبتة *A. armatus* بولاية قسنطينة، دائرة بكيرا (شرق الجزائر) في مايو 2007. تم الاحتفاظ بعينة النبتة في معشبة كلية العلوم (جامعة قسنطينة -1) تحت الرقم LOST Aa.05.07 (Khalfallah *et al*., 2014).

تم إجراء استخلاص الجزء العلوي في مختبر التحصيل للمواد العلاجية (LOST) التابع لجامعة قسنطينة -1. تخضع المرحلة المائية لاستخلاص نوع سائل-سائل باستخدام مذيبات ذات قطبية متزايدة بدءًا من الكلوروفورم، ثم أسيتات الإيثيل وأخيرًا البيوتانول.

2. الحيوانات:

الحيوانات المستعملة في هذه الدراسة هي فئران تجارب من نوع *Mus musculus*، تم الحصول عليها من معهد الصيدلة بقسنطينة، أما التجارب فأجريت على الحيوانات بعد أن أعطيت فترة للتأقلم مع ظروف العمل المخبري قبل كل تجربة.

3. التجارب:

تم إجراء البحث على 28 فأرًا بالغًا من فئران *Mus Musculus*، تتراوح أعمارها بين 2.5 و 3 أشهر، وتتراوح أوزانها بين 30 و 35 غ. تم الحصول على جميع الحيوانات من الصيدلية المركزية بالجزائر وتم إيواؤها في أقفاص بلاستيكية. بعد إنقضاء فترة التكيف، قسمت الفئران إلى 4 مجموعات بحيث تحتوي كل مجموعة على 7 فئران، مع توفير الماء والغذاء لمدة 21 يومًا مع التحكم في النظام الغذائي التجريبي (الجدول 01).

الجدول 01 : المعاملة التجريبية للفئران.

المجموعة التجريبية	المادة المستخدمة	عدد الفئران	الجرعة اليومية	المدة التجربة
المجموعة (F)	مسحوق الدقيق اللين	7	0.5 مغ/ فأر	21 يوم
المجموعة (M)	مسحوق الدقيق اللين+ الميثيونين	7	0.5 مغ/ فأر + 400 مغ/كغ	21يوم
المجموعة (MP)	مسحوق الدقيق اللين+ الميثيونين+ مستخلص النبتة	7	0.5 مغ/ فأر + 400 مغ/كغ + 100 مغ/كغ	21 يوم
المجموعة (P)	مسحوق الدقيق اللين + مستخلص النبتة	7	100مغ/كغ	21 يوم

3. 1. أخذ عينة الدم و الأنسجة:

بعد 21 يومًا من المعاملة التجريبية الموضحة في الجدول السابق، تم منع الغذاء عن الحيوانات طوال الليل و تم تخذيرها، ثم أخذت عينات الدم من الوريد بجانب العين و وضعت في أنابيب EDTA و تم طردها مركزيًا، بعدها قمنا بتخزين البلازما في -20 درجة مئوية حتى القيام بالتحليل الكيميائي. و تم إزالة الشريان الأورطي والقلب على الفور وغسلهما بمحلول PBS الملحي، بعدها تم تجميد جزء من كبد كل حيوان لمعايرة نسبة مضادات الأكسدة الإنزيمية و المتمثلة في الجلوثاتيون (Gluthatione) و الكاتلاز(Catalase) ، بينما تم

استخدام أجزاء أخرى من الشريان الأورطي وقلب كل حيوان و جزء من الكبد في الدراسة النسيجية. أجريت التجارب في إطار الإمتثال الصارم للمبادئ الأخلاقية التي وضعتها لجنة CPCSEA لغرض المراقبة والإشراف على تجارب الحيوان.

3. 2. التحاليل البيوكيميائية:

تم قياس كل من الهوموسيستيين، الدهون الثلاثية، الكوليسترول، البروتينات الدهنية منخفضة الكثافة، البروتينات الدهنية عالية الكثافة في مخبر التحاليل البيولوجية " إبن سينا" بقسنطينة.

3. 3. معايرة نشاط مضادات الأكسدة:

3. 3 .1. قياس البروتين:

تم تحديد تركيزات البروتين من خلال طريقة برادفور (Bradford, 1976)، تم تحديد تركيز البروتين من خلال مقارنته بخط المعايرة BSA .

3. 3 .2. قياس الجلوثاتيون المختزل Gluthatione:

تم تحديد تركيز الجلوتاثيون في الكبد بواسطة طريقة (Weckbercker and Cory, 1988). بعد إنقضاء فترة التجربة، تم تخدير الحيوانات و تشريحها لنزع الكبد بصفة فورية، في حالته الرطبة، بعد ذلك تم إستخدام مسحوق الكبد المتجانس لمعايرة كمية الجلوثاتيون بإستعمال كاشف التلوين DTNB، بعد مرور 5 دقائق تم قياس الكثافة الضوئية عند 412 نانومتر على مقياس طيف ضوئي.

تم حساب تركيز الجلوتاثيون بالصيغة التالية:

$$GSH\ (nmol/mg\ of\ protein) = \frac{OD \times 1 \times 1.525}{13100 \times 0.8 \times 0.5.mg\ protein}$$

حيث:

OD : الكثافة الضوئية

1: الحجم الكلي للحلول في نزع البروتين

1.525: الحجم الكلي للحلول المستخدمة في فحصGSH

13100: معامل الامتصاص عند التجميع SH — حتى 412 نانومتر

0.8: حجم عينة المجانسة homogenat sample

0.5: حجم طاف supernatant

3. 3 .3. قياس نشاط الكتالاز Catalase:

تم تقييم نشاط الكاتلاز في مسحوق الكبد المتجانس وفقًا لطريقة (Aebi, 1974)، فهذا الإنزيم له القدرة على تحليل بروكسيد الهيدروجين H2O2 إلى جزيئة ماء و أكسجين، تم قياس نشاط الكاتلاز طيفيا.

يُعتقد أن التفاعل يحدث على مرحلتين:

$$\text{Catalase-(Fe III)} + H_2O_2 \longrightarrow [\text{Catalase-H 2O-(Fe V)}] + H_2O$$

$$[\text{Catalase-H 2O-(Fe V)}] + H_2O_2 \longrightarrow \text{Catalase-(Fe III)} + H_2O + O_2$$

$$2\ H_2O_2 \longrightarrow 2\ H_2O_2 + O_2$$

حدد نشاط الكاتلاز عن طريق قياس الانخفاض في الامتصاصية عند 240 نانومتر لمدة دقيقة واحدة (15 إلى 60 ثانية) نتيجة لاستهلاك H2O2. الطريقة المتبعة موضحة في الجدول التالي:

الجدول :02

الكواشف	العينات(µL)	الشاهد (µL)
محلول الفوسفات µM100، (pH=7.5)	790	800
H_2O_2	200	200
السائل الطافي لمسحوق الكبدالمتجانس	10	-

بعدها تم تقدير فعالية الإنزيم كما هو موضح في العلاقة التالية:

$$\text{Catalase activity (mmol H2O2/min/ mg prot)} = \frac{\Delta DO}{\varepsilon \times L \times \chi \times Fd}$$

حيث:

ΔD:الكثافة الضوئية

ε: معامل الإمتصاصية المولية

L : طول كوفيت المستعملة

χ: كمية البروتين

Fd :عامل التخفيف.

3 .4. تحضير القطاعات النسيجية:

بعد جمع عينات الدم لإجراء التحاليل البيوكيميائية، تم تشريح الفئران و جمع عينات كل من الشريان الأورطي، القلب والكبد لإجراء فحوصات مجهرية ضوئية حسب المراحل التالية .

- تم شطف العينات من جميع الأنسجة الملتصقة بمحلول ملحي بالفوسفات (0.9٪) بعد ذلك، يتم تخزينها في قوارير صغيرة تحتوي على الفورمول المخفف بنسبة 10٪ بغرض تثبيتها.
- وضعت الأجزاء المختلفة من الشريان الأورطي في محلول بوين الكحولي لمدة 5 دقائق للتلوين (بسبب لونها الشفاف).
- بعد ذلك تم نزع الماء بإستخدام أحواض كحول الإيثانول بتراكيز متزايدة (50%، 75% و 96%)، مدة كل مرحلة حوالي 30 د، تعاد كل مرحلة 3 مرات، ثم تخزن في البيوتانول لمدة ثلاثة أيام.
- توضع في محلول كزيلين لمدة 20 دقيقة (يتم تغيير الحمام كل 10 دقائق).
- الطمر في شمع البارافين: ثلاثة حمامات بارافين (مدة كل حمام ساعة واحدة و 30 دقيقة، يتم الحفاظ على درجة حرارة الفرن عند 60 درجة مئوية.

- تشكيل كتل البارافين المحتوية على العينة المراد دراستها ثم تقطع العينات بالمقطاع المجهري ليتم تلوينها بصبغة (Hematoxylin eosin).
- بعد التجفيف على الصفيحة الساخنة (50 درجة مئوية)، تم وضع العينة بين الشريحة والساترة .
- تتم القراءة بواسطة مجهر ضوئي متصل بالكمبيوتر.

3 .5. التحليل الإحصائي:

تم تقديم التحليلات الكيميائية الحيوية لكل من الهوموسيستيين، الدهون و إنزيمات المضادة للأكسدة في شكل وسائل مصحوبة بالخطأ المعياري (mean ± SEM)، و تم إجراء المقارنة بين المجموعات بواسطة اختبار أحادي الاتجاه ANOVA.

ثانيا: تقييم النشاط المضاد للأكسدة للكسر البوتانولي (n-BuOH) لنبتة *A. armatus*

1. اختبار القدرة الكلية المضادة للأكسدة:

تم قياس القدرة الكلية المضادة للأكسدة للمستخلص باتباع طريقة الفوسفوموليبدينوم phosphomolybdenum (Prieto *et al*., 1999).

و يتكون الكاشف من:

- مولبيدات الأمونيوم 4 mM
- حمض الكبريت M0.6
- فوسفات الصوديوم 28 mM

تمت قراءة الامتصاصية لعينة الفسفوموليبدينوم الناتجة عن الامتصاص عند 695 نانومتر وفقا لطريقة العالم (Ozen *et al*., 2017).

تم استخدام BHA و BHT وحمض الأسكوربيك كعناصر تحكم إيجابية.

2. اختبارالقدرة الإختزالية:

في هذا الاختبار، تؤدي مضادات الأكسدة المختزلة إلى تقليل مركب (Ferricyanide) Fe^{+3} إلى شكل

Fe^{+2}ومراقبتها عن طريق قياس الإمتصاصية عند 700 نانومتر(Oyaizu *et al.*, 1986).

يشار إلى أن الإمتصاص العالي للعينة له قدرة تخفيض فعالة في حالة التفاعل التي زادت فيها قدرة الاختزال. تم استخدام BHA،BHT ، trolox و حمض الأسكوربيك كعناصر تحكم إيجابية.

3. اختبار القدرة على إقتناص الجذور الحرة ($DPPH^{\cdot}$) :

تم قياس أنشطة التبرع الإلكتروني لمحلول العينة بطريقة القياس الطيفي عن طريق تبييض المحلول ذي اللون الأرجواني لنشاط مسح الجذور الحرة وفقًا لتقنية (Blois, 1958).

تمت مراقبة امتصاص الخليط عند 517 نانومتر. استخدمت كل من BHA ، BHT ، trolox و حمض الأسكوربيك كعناصر تحكم إيجابية.

تم حساب النشاط كنسبة مئوية من تغير لون DPPH ، باستخدام المعادلة:

Free radical scavenging activity % = [(A532 (blank) - A532 (sample))⁄A532 (blank)] × 100.

4. اختبارالقدرة على استخلاص المعادن:

تم قياس القدرة على استخلاص المعادن للمستخلص باتباع طريقة (Dinis *et al.*, 1994) ، باستخدام EDTA كعنصر تحكم إيجابي. تمت قراءة الإمتصاص عند 562 نانومتر. و تم حساب النشاط كنسبة مئوية من تثبيط تكوين مركب فيروزين + Fe2 وفقًا للصيغة:

Metal chelating activity % = [(A562 (blank) - A562 (sample))⁄A562 (blank)] × 100

ثالثا: التأثير الوقائي للكسر البوتانولي (n-BuOH) لنبتة *A. armatus* على النمو والنشاط التكاثري لبعض الخلايا السرطانية مثل خلايا HeLa وخلايا C6

1. تحضير وسط الزرع:

تم تطوير خلايا HeLa (سرطان عنق الرحم البشري) وخلايا C6 (ورم دماغ الجرذان) في محلول DMEM-HG مع 10٪ FBS و 2٪ بنسلين (ستربتوميسين-ج)، في جو رطب بنسبة 5٪ من ثاني أكسيد

الكربون، في مختبر أبحاث النبات، قسم الكيمياء في جامعة شانكيري كاراتكين، تركيا. بعدها فصلت خلايا HeLa و C6 من دورق المزرعة باستخدام 10 مل من خليط التربسين EDTA .تمت إضافة نفس الحجم من وسط الزرع وخلطه جيدًا، بعدها تمت عملية الطرد المركزي بواسطة جهاز (Nüve NF 800 ، تركيا). تم خلط 5 مل من الوسط مع المادة الطافية. و تم تحديد تركيز الخلايا في هذا المعلق باستخدام جهاز CEDEX HIRES Cell Counter (Demirtas and Sahin, 2012).

2. اختبارات تكاثر الخلايا:

تم زرع الخلايا في أطباق زراعة مكونة من 96 طبق بكثافة 30.000 خلية في كل طبق (COSTAR, Corning, USA). تمت دراسة نشاط العينات عند التراكيز 100، 75، 50، 40، 30، 20، 10 و 5 ميكروغرام/مل. استخدم FU-5 كمركبات قياسية standard compounds. ثم تم تحضين الخلايا طوال الليل قبل تطبيق كاشف اختبار ELISA assay reagent لتكاثر الخلايا BrdU، حددت نسبة تكاثر الخلايا A450 نانومتر باستخدام قارئ الألواح الدقيقة (Awareness Chromate, USA)، تم تدوين النتائج كنسبة مئوية من تثبيط تكاثر الخلايا. حسبت النسبة المئوية لتثبيط تكاثر الخلايا وفقا للصيغة:

[(1-Atreatments / Avehicle control)] x 100.

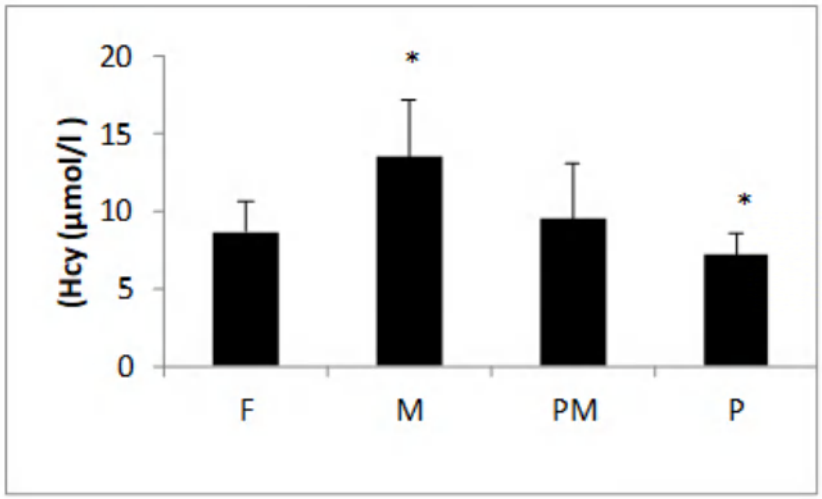
تم اختبار الاختلافات بين المجموعات باستخدام برنامج ANOVA و اعتبرت قيم $p<0.01$ مهمة وتم تحليلها بواسطة برنامج

SPSS (version 11.5 for Windows 2000, SPSS Inc)، (Sahin *et al*., 2014).

النتائـــــج

النتائج المتحصل عليها:

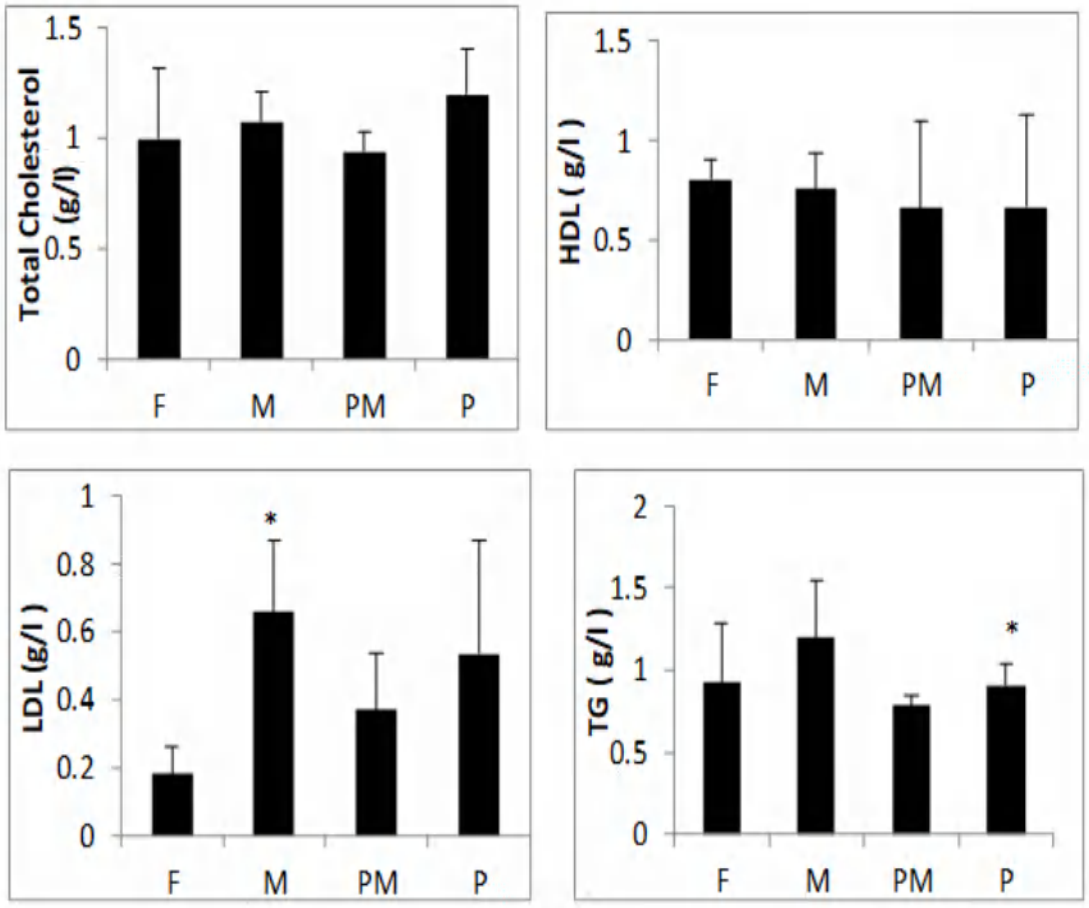
- بينت النتائج المتحصل عليها ارتفاعا في الهوموسيستين في المجموعة M التي أعطيت جرعات عالية من الميثيونين (400 مغ/كغ) مقارنة مع المجموعة F، وهي مجموعة الشاهد التي تناولت غذاءا عاديا يحتوي على مسحوق الدقيق (0.5 مغ/ فأر) فقط ، بينما لاحظنا انخفاضا معتبرا في نسبة الهوموسيستيين لدى المجموعة MPالتي تناولت جرعات عالية من الميثيونين (400 مغ/كغ) بالإضافة إلى مستخلص نبتة *A. armatus* الشكل (1) ، هذا ما يثبت نجاعة مستخلص النبتة في الوقاية و الحد من ارتفاع الهوموسيستيين في الدم.



الشكل 1: تأثير L-methionine و مستخلص *A. armatus* على الهوموسيستيين Hcy في الفئران خلال 21 يومًا من العلاج، (F) مجموعة الشاهد، (M) المجوعة المعالجة بـ L-methionine، (MP) المجوعة المعالجة بمستخلص L-methionine و *A. armatus*، (P) المجوعة المعالجة بمستخلص *A. armatus*.

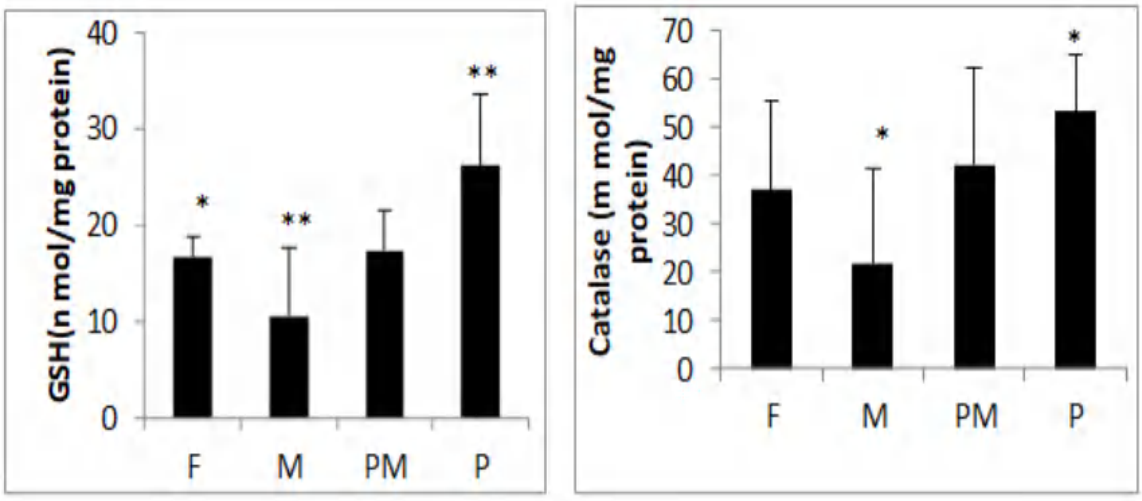
- أظهرت النتائج المتحصل عليها بالنسبة للتحاليل البيوكيميائية لكل من الكولسترول، الكولسترول المنخفض الكثافة (LDL-c) و الدهون الثلاثية (TG) ارتفاعا بقيمة معتبرة، بينما انخفضت في البروتينات الدهنية عالية الكثافة (HDL-c) بقيمة معتبرة لدى المجموعة M التي تناولت جرعات عالية من الميثيونين (400 مغ/كغ) و ذلك مقارنة مع مجموعة الشاهد F التي تناولت غذاءا عاديا يحتوي على مسحوق الدقيق (0.5 مغ/ فأر).

- بينما بينت النتائج انخفاضا معتبرا لكل من الكولسترول، الكولسترول المنخفض الكثافة(LDL-c) ، الدهون الثلاثية (TG) و البروتينات الدهنية عالية الكثافة (HDL-c) في المجموعة MP التي تناولت جرعات عالية من الميثيونين بالإضافة إلى مستخلص نبتة *A. armatus* الشكل (2)، مما يثبت دور و فعالية مستخلص النبتة في خفض مستويات الدهون في الدم.



الشكل 2: تأثير L-methionine و مستخلص *A. armatus* على الليبيدات (الكولسترول، الكولسترول المنخفض الكثافة (LDL-c) و الدهون الثلاثية (TG) ،البروتينات الدهنية عالية الكثافة (HDL-c) في الفئران، خلال 21 يومًا من العلاج، (F) مجموعة الشاهد، (M) المجوعة المعالجة بـL-methionine ، (MP) المجوعة المعالجة بمستخلص L methionine و*A. armatus* ، (P) المجوعة المعالجة بمستخلص *A. armatus*

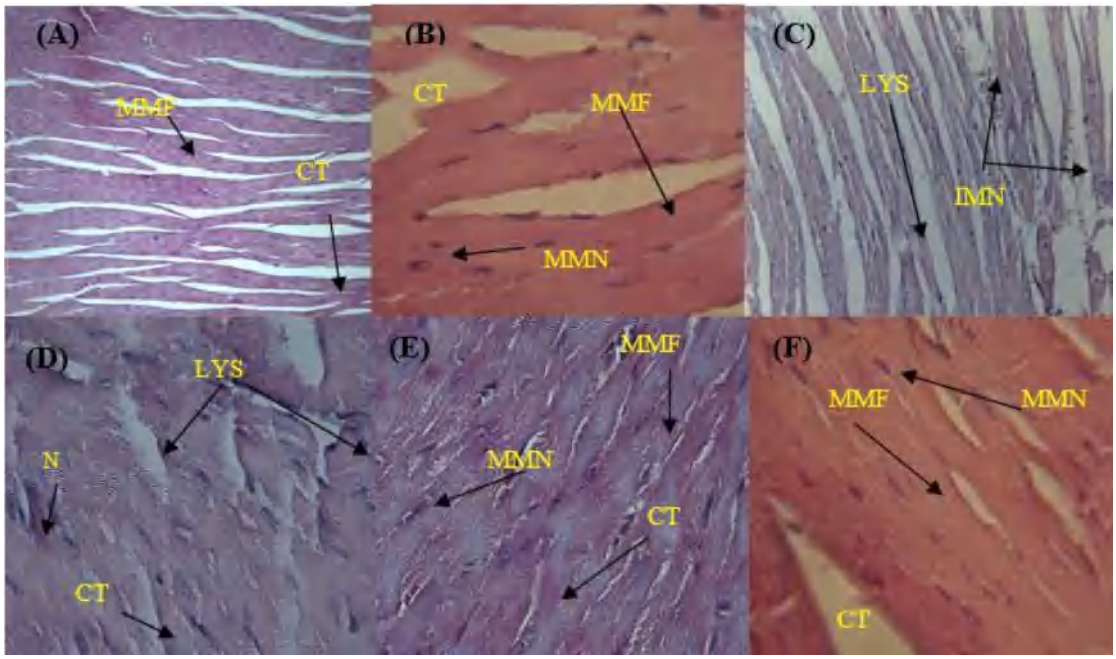
- بالنسبة لنشاط مضادات الأكسدة بينت النتائج إنخفاضا بقيمة معتبرة لكل من الجلوثاتيون المختزل و الكاتالاز في المجموعة M التي تناولت جرعات عالية من الميثيونين (400 مغ/كغ) و ذلك مقارنة مع مجموعة الشاهد F التي تناولت غذاءا عاديا يحتوي على مسحوق الدقيق 0.5 مغ/ فأر و أيضا باقي المجموعات، في حين بينت المجموعة MPالتي تناولت جرعات عالية من الميثيونين بالإضافة إلى مستخلص نبتة *A. armatus* إرتفاعا معتبرا لكل من الجلوثاتيون المختزل و الكاتالاز الشكل (3).



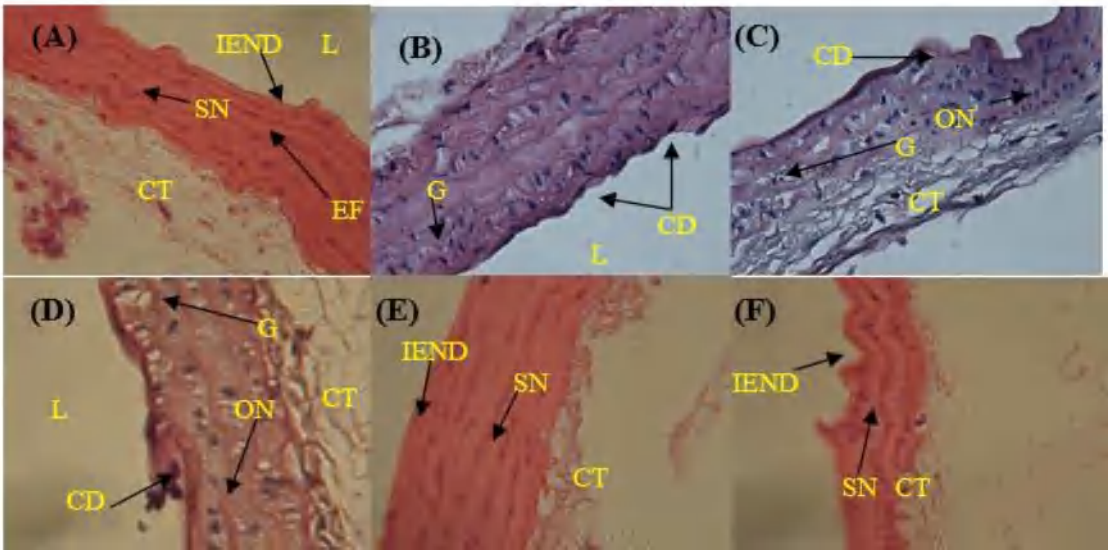
الشكل 3: تأثير L-methionine و مستخلص *A. armatus* على مضادات الأكسدة الإنزيمية (الجلوثاتيون و الكاتالاز) في الفئران خلال 21 يومًا من العلاج، (F) مجموعة الشاهد، (M) المجوعة المعالجة بـ-L methionine، (MP) المجوعة المعالجة بمستخلص L-methionine و*A. armatus* ، (P) المجوعة المعالجة بمستخلص *A. armatus*

- أظهرت الدراسة المجهرية للمقاطع النسيجية لدى المجموعة M التي تناولت جرعات عالية من الميثيونين (400 مغ/كغ) تحلل ونخر في بنية الألياف العضلية وتسلل الخلايا وحيدة النواة بين خلايا العضلات بالنسبة لنسيج القلب الشكل(4) ، بينما بينت المقاطع النسيجية لشريان الأورطى تقشر البطانة الداخلية، لاحظنا أيضًا ظهور تحلل، وتشكل الخلايا الرغوية والنواة البيضاوية في الطبقة الوسطى الشكل(5) ، أيضا بالنسبة لنسيج الكبد فقد لاحظنا تلف النسيج الكبدي الشديد الذي يتميز بالنخر، وتغير في شكل نوى الخلايا الكبدية مع ظهور حويصلات ليبيدية

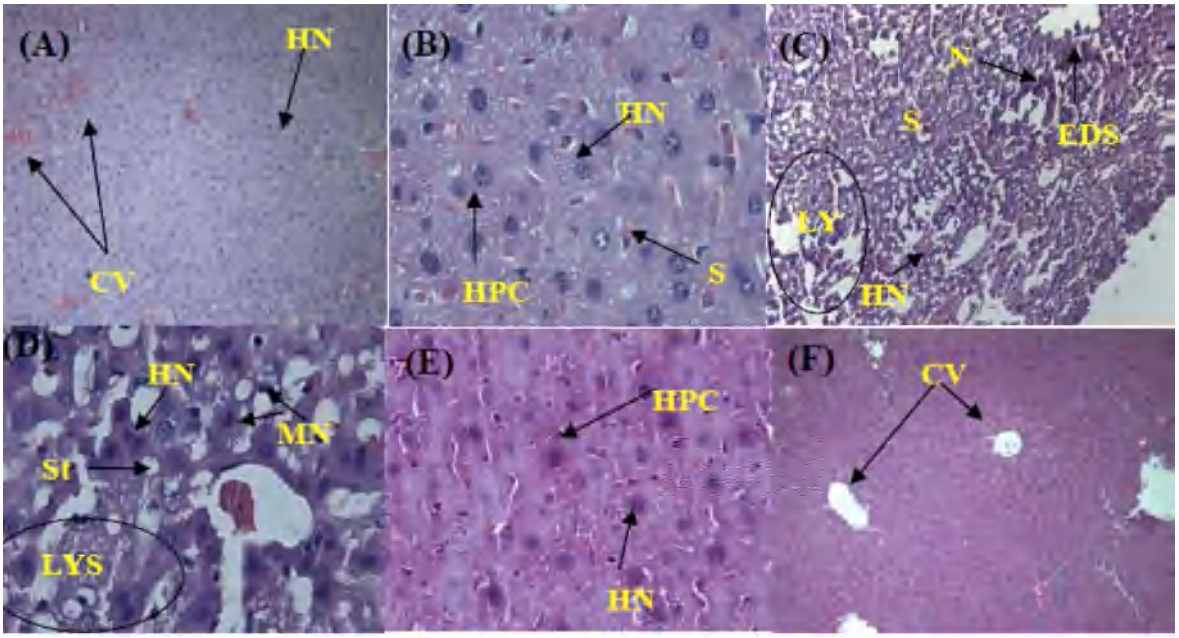
التي أدت إلى التشحم الكبدي و ذلك مقارنة مع المجموعات الأخرى الشكل(6). من جهة أخرى بينت الدراسة المجهرية للمقاطع النسيجية لدى المجموعة MP التي تناولت جرعات عالية من الميثيونين بالإضافة إلى مستخلص نبتة *A. armatus* سلامة أنسجة كل من القلب، شريان الأورطى و الكبد.



الشكل 4: ملاحظة مجهرية لنسيج القلب، خلال 21 يوما من العلاج، (A و B) مجموعة الشاهد، (C و D) المجموعة المعالجة بالميثيونين، (E) المجموعة المعالجة بالميثيونين و مستخلص نبتة *A. armatus*، (F) المجموعة المعالجة بمستخلص نبتة *A. armatus*.



الشكل 5: ملاحظة مجهرية لنسيج شريان الأورطى، خلال 21 يوما من العلاج، (A) مجموعة الشاهد، (C، B و D) المجموعة المعالجة بالميثيونين، (E) المعالجة بالميثيونين ومستخلص نبتة *A. armatus*، (F) المجموعة المعالجة بمستخلص نبتة *A. armatus*.



الشكل 6: ملاحظة مجهرية لنسيج الكبد، خلال 21 يوما من العلاج، (A و B) مجموعة الشاهد، (C و D) المجموعة المعالجة بالميثيونين، (E) المعالجة بالميثيونين ومستخلص نبتة *A. armatus*، (F) المجموعة المعالجة بمستخلص نبتة *A. armatus*

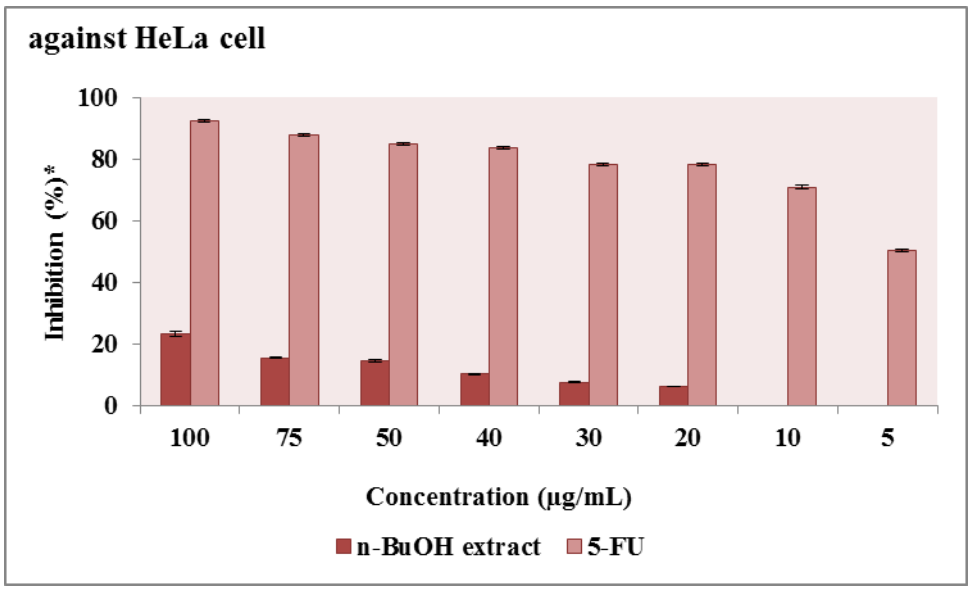
- في دراسة أخرى لتقييم النشاط المضاد للأكسدة للكسر البوتانولي لنبتة *A. armatus* بينت النتائج المتحصل عليها بأربع طرق: اختبار القدرة الكلية المضادة للأكسدة، اختبارالقدرة الإختزالية، اختبار القدرة على إقتناص الجذور الحرة (DPPH˙)، اختبارالقدرة على استخلاص المعادن، أظهرت نشاطًا وقدرة منخفضة في الفعل المضاد للأكسدة الكلية، القدرة الإختزالية، القدرة على إقتناص الجذور الحرة، استخلاص المعادن لهذا المستخلص، كما هو موضح في الجدول (3).

الجدول (3): تقييم النشاط المضاد للأكسدة للكسر البوتانولي لنبتة *A. armatus*

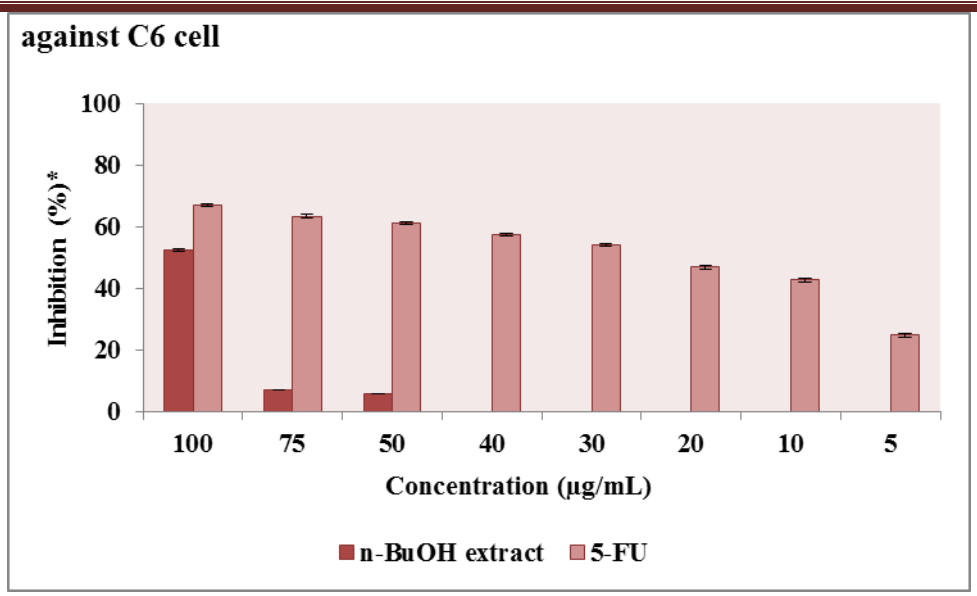
Samples	In vitro Antioxidant Assays			
	50 µg mL-1	100 µg mL-1	250 µg mL-1	500 µg mL-1
Total antioxidant activity, 695 nm				
n-BuOH Aa extract	0.141±0.023	0.180±0.015	0.341±0.037	0.593±0.075
BHA	0.228±0.020	0.469±0.024	0.702±0.019	1.699±0.046
BHT	0.199±0.032	0.304±0.032	0.537±0.002	0.875±0.036
Ascorbic acid	0.251±0.030	0.477±0.015	1.097±0.051	2.680±0.052
Reducing power, 700 nm				
n-BuOH Aa extract	0.086±0.005	0.087±0.015	0.123±0.016	0.143±0.004
BHA	0.265±0.006	0.334±0.014	0.429±0.092	0.465±0.032
BHT	0.269±0.015	0.350±0.011	0.438±0.024	0.564±0.041
Ascorbic acid	0.191±0.048	0.327±0.002	0.366±0.028	0.379±0.031
Trolox	0.213±0.001	0.296±0.006	0.367±0.033	0.386±0.035
Free radical scavenging activity (DPPH˙), %				
n-BuOH Aa extract	5.80±1.85	24.43±5.76	30.56±4.42	48.26±3.99
BHA	69.48±3.64	77.46±4.61	85.17±5.38	89.29±2.10
BHT	10.02±5.52	25.49±5.89	35.28±8.30	43.38±3.51
Ascorbic acid	74.91±7.43	82.27±6.45	80.05±2.29	84.68±4.81
Trolox	73.83±2.46	86.75±6.18	89.13±4.48	94.24±2.85
Metal chelating activity, %				
n-BuOH Aa extract	5.53±2.83	6.19±1.82	12.88±2.37	31.08±3.52
EDTA	52.27±6.18	94.01±6.86	97.72±1.47	98.96±0.54

47

- أما الدراسة الأخيرة والتي تتمثل في التأثير الوقائي للكسر البوتانولي لنبتة *A. armatus* على النمو والنشاط التكاثري لبعض الخلايا السرطانية مثل خلايا HeLa وخلايا C6، فإن استعمال تراكيز مختلفة من هذا المسخلص يؤدي إلى تثبيط النمو والنشاط التكاثري لبعض الخلايا السرطانية مثل خلايا HeLa وخلايا C6 بالإضافة إلى ذلك، تم تقييم مستخلص الكسر البوتانولي لنبتة *A. armatus* أنه يحتوي على أنشطة عالية مضادة للتكاثر ضد خلايا C6 مقارنة بخلايا HeLa عند التركيز 100 ميكروغرام/مل كما هو موضح في الأشكال (7، 8).



الشكل (7): النشاط المضاد للتكاثر للكسر البوتانولي لنبتة *A. armatus* و5-FU مقابل خط خلايا HeLa



الشكل (8): النشاط المضاد للتكاثر للكسر البوتانولي لنبتة *A. armatus* و5-FU مقابل خط خلايا C 6.

المناقشة

1-تأثير أخذ جرعات عالية من الميثيونين على مستويات الهوموسيستيين، الدهون، نشاط مضادات الأكسدة الإنزيمية و الدور الوقائي لمستخلص نبتة *A. armatus*

كما ذكرنا في البداية، يتم انتاج الهوموسيستين من الميثيونين L-methionine عند البشر و الحيوانات، و لأننا نحترم هذا المسار الأيضي، فقد أعطينا الفئران L-methionine بدلاً من homocysteine ، لذلك فالهدف الرئيسي لعملنا هو توضيح آثار ارتفاع جرعات عالية من الميثيونين على مستويات (الهوموسيستيين، الدهون، مضادات الأكسدة الإنزيمية الجلوثاتيون و الكاتالاز)، وعلى التركيب النسيجي لأعضاء معينة هي (الشريان الأورطي، القلب والكبد) في الفئران. بالإضافة إلى ذلك قمنا بتقييم التأثير العلاجي و الوقائي لمستخلص نبتة *A. armatus* على التغيرات الناتجة عن جرعة عالية من L-methionine (400 مغ / كغ) خلال 21 يومًا من العلاج.

يعتبر فرط هوموسيستيين الدم، الذي يتميز بارتفاع مستويات الهوموسيستيين في البلازما، عامل خطر مرتبط بأمراض القلب والأوعية الدموية (Boyacioglu *et al*., 2014)، ويعتقد أنه يتسبب في الإجهاد التأكسدي الداخلي ويسبب العديد من الأضرار الخلوية (Derouiche *et al*., 2014).

يلعب نقص حمض الفوليك والفيتامينات B6 و B12 دوراً هاماً في تطور حالة فرط الهوموسيستيين في الدم (Domagala *et al*., 1997).

بينت العديد من الدراسات أنه يمكن الوقاية من فرط هوموسيستيين الدم بواسطة مركبات البوليفينول، وهي مركبات قوية مضادة للأكسدة ذات خصائص مضادة لتصلب الشرايين (Noll *et al*., 2011).

إن استخدام الفيتامينات لتعديل استقلاب الهوموسيستين يقلل بشكل كبير من المخاطر عن طريق تقليل مستويات الهوموسيستين في البلازما (Sim *et al*., 2016).

أظهرت النتائج المتحصل عليها أن النظام الغذائي ذو جرعات عالية من الميثيونين (400 مغ/كغ) للفئران لمدة 21 يوم، قد أدى إلى إرتفاع مستويات كل من الهوموسيستيين و الدهون، بينما عرفت كل من البروتينات الدهنية عالية الكثافة (HDL-c) و مضادات الأكسدة الإنزيمية (الجلوثاتيون و الكاتالاز) إنخفاضا معتبرا، هذا ما أدى إلى إلحاق أضرار في البنية النسيجية لكل من القلب، الشريان الأروطي و الكبد.

نتائجنا بينت أن تناول جرعات عالية من الميثيونين (400 مغ/كغ) للفئران لمدة 21 يوم أدت إلى ظهور حالة فرط هوموسيستيين الدم، تتوافق النتائج التي تم الحصول عليها مع الدراسة التجريبية السابقة لكل من (Boyacioglu *et al.*, 2014; Derouiche *et al.*, 2014) والتي أظهرت أن استهلاك نظام غذائي عالي الميثيونين تسبب في زيادة كبيرة في نسبة الهوموسيتيين في البلازما. يؤدي فرط الهوموسيتيين في الدم إلى أعراض مرضية متنوعة، ويُعتقد أن السبب الذي يجعل الهوموسيستين عاملا ممرضا هو قدرته على إنتاج و زيادة الإجهاد التأكسدي (Noll et *al.*,2011) .

علاوة على ذلك، اكتشفنا زيادة في تركيز الدهون التالية (Tch) ،(LDL-c) و(TG)، بينما عرفت كل من البروتينات الدهنية عالية الكثافة (HDL-c) و مضادات الأكسدة الإنزيمية (الجلوثاتيون و الكاتالاز) إنخفاضا معتبرا بعد تناول الميثيونين (400 مغ/كغ) بجرعات عالية لمدة 21 يوم، هذا يتفق مع الدراسة التجريبية السابقة من قبل (Obeid *et al.*, 2009; Momin *et al.*, 2017; Shaker *et al.*, 2013)، وقد اقترح وجود ارتباط بين ارتفاع الدهون في الدم و حالة فرط الهوموسيستيين. و مع ذلك بين (Obeid *et al.*, 2009) في دراسته أن حالة فرط الهوموسيستيين يعمل على تثبيط نقل الكولسترول العكسي عن طريق تقليل HDL-c المنتشر و يتم ذلك من خلال تثبيط تخليق البروتين apoA-I وتعزيز تصفية HDL-c.

تكون الزيادة في LDL مرتبطة بأكسدتها بواسطة الجذور الحرة الناتجة عن ارتفاع الهوموسيستيين، لأن هذا البيروكسيد يمنع التعرف على LDL المؤكسد بواسطة مستقبلاته الأصلية (Laporte, 2000). أثبتت دراسة (Werstuck *et al.*, 2001) أن تراكم الكوليسترول والدهون الثلاثية في الكبد الناتج عن حالة ارتفاع الهوموسيستيين يرتبط بالزيادة في التخليق الحيوي والإمتصاص لهذه الدهون.

من جهة أخرى، أظهرت نتائجنا انخفاضًا في أنشطة مضادات الأكسدة الإنزيمية الجلوثاتيون و الكاتالاز GSH، CAT في الفئران عند إعطاء جرعة عالية من الميثيونين. هذا ما يتوافق مع الدراسة التالية التي أثبتت أن الهوموسيستيين يعمل على التقليل من نشاط مضادات الأكسدة الأنزيمية في الأنسجة، و تلعب الجذور الحرة دورًا مهمًا في هذه الحالة الناتجة عن فرط الهوموسيستيين في الدم (Boyacioglu *et al.*, 2014)، هذا لأن العلاج بالميثيونين طويل الأمد يعزز الإجهاد التأكسدي، لأنه يقلل من الدفاعات المضادة للأكسدة غير الأنزيمية،

ويزيد من أكسدة الدهون والبروتين الكربوني، ويغير نشاط إنزيمات مضادات الأكسدة، ويغير من التحاليل البيوكيميائية في الدم (Stefanello *et al*., 2009 ; Matté *et al*., 2009) .

تعد أمراض القلب والأوعية الدموية بالإضافة إلى أمراض الكبد من المشكلات الرئيسية للصحة العامة، و قد ارتبط ارتفاع الهوموسيستيين في الدم بأمراض القلب والأوعية الدموية، ويعد الخلل في استقلاب مجموعة الميثيل من بين الأحداث الجزيئية الرئيسية التي يُعتقد أنها تلعب دورًا في إصابة الكبد (Selicharová *et al*., 2013). حسب دراسة (Boyacioglua *et al*., 2014)، فإن تأثير الجذور الحرة للأكسجين، الناتجة عن ارتفاع الهوموسيستيين في الدم لا يكون على مستوى أنسجة القلب فحسب، بل أثرت أيضًا على أنسجة الكبد و الكلى.

بينت دراستنا أن الفحوصات النسيجية المرضية في الفئران ذات فرط الهوموسستيين في الدم أدى إلى ظهور تغيرات نسيجية في الشريان الأورطي، القلب و الكبد، وقد لوحظ ذلك من خلال التحلل والنخر في بنية الألياف العضلية، وتسلل الخلايا أحادية النواة بين الخلايا العضلية في أنسجة القلب. شريان الأبهر أظهر تقشر الخلايا الطلائية، ولاحظنا أيضًا تشكل الفجوات، وتكوين الخلايا الرغوية والأنوية البيضاوية.

نتائجنا تتفق مع دراسة (Aklil *et al*., 2017; Benmebarek *et al*., 2013) التي أكدت أن تناول الميثيونين عن طريق الفم بكميات عالية لدى الفئران يمارس نشاطًا سامًا على الشريان الأورطي وله تأثير سام على القلب.

بالإضافة إلى ذلك ، فإن ملاحظتنا المجهرية لنسيج الكبد في الفئران التي عولجت بكميات عالية من الميثيونين كشفت عن تلف الكبد الكلي الذي يتميز بالنخر، وتغير في شكل نوى الخلايا الكبدية وظهور حالة التشحم الكبدي.

تتفق نتائجنا مع دراسة (Benmebarek *et al*., 2013; Taravati *et al*., 2013)، التي أفادت بأن فرط الهوموسستيين في الدم يكون تأثيره سامًا على أنسجة الكبد.

يعمل فرط الهوموسستيين في الدم عن طريق الإجهاد التأكسدي على تعزيز تليف عضلة القلب والخلل الوظيفي للقلب (Joseph *et al*., 2008). سبب ذلك أن زيادة الإجهاد التأكسدي في حالة فرط الهوموسستيين يلعب دورًا فيزيولوجيًا مرضيًا في البطانة الداخلية للأوعية الدموية مما يؤهب الأوعية المصابة للتطور اللاحق

للإصابة بتصلب الشرايين (Kanani *et al*., 1999).

في درسة سابقة، تم الإشارة إلى وجود ارتباط بين وجود تمدد الأوعية الدموية للشريان الأورطي وارتفاع مستويات الهوموسيستين في الدم ، حيث يلعب الهوموسيستين دورًا مهمًا في تطور إنسلاخ هذا الشريان (Takagi *et al*., 2005).

تظهر نتائج (Lamda *et al*., 2014) أن ارتفاع الهوموسيستين في الدم يزيد من تخليق الكوليسترول، ويمارس تأثيرًا سامًا للأوعية الدموية خاصة الشريان الأورطي من خلال فقدان البطانة، والتحلل جزئيًا مع انحلال خلايا الطبقة المتوسطة.

أشار التحليل النسيجي للكبد إلى وجود التسلل الإلتهابي، التليف وانخفاض محتوى الجليكوجين/البروتين السكري في الفئران المصابة بفرط الهوموسيستين (Matte´*et al*., 2009). ينتج ارتفاع الهوموسيستين في الدم عن خلل في التمثيل الغذائي الكبدي ويتميز بارتفاع مستوى هوموسيستين البلازما، مما يؤدي إلى آفات كبدية واستقلاب غير طبيعي للدهون (Latour *et al*., 2015).

من جهة أخرى، وجدنا أن العلاج بمستخلص نبتة *A. armatus* كان فعالًا في الحماية ضد ارتفاع الهوموسيستين في الفئران التي أخذت نظامًا غذائيًا غنيًا بـالميثيونين لمدة واحد و عشرون يوما عن طريق خفض مستويات الهوموسيستين في البلازما، وتقليل نسبة الدهون مع تقليل الإجهاد التأكسدي عن طريق زيادة تركيزات مضادات الأكسدة الإنزيمية GSH و CAT بالإضافة إلى حماية أنسجة الشريان الأورطي، القلب و الكبد.

نتائجنا تتفق مع الدراسة التجريبية السابقة التي أجراها كل من

(Aklil *et al*., 2017; El-Saleh *et al*., 2004; Haddadi *et al*., 2017)، حيث أفاد هؤلاء أن ارتفاع الهوموسيستين ناتج عن الجرعة العالية من الميثيونين في الفئران والجرذان، وأكدوا الدور الوقائي الكبير لمستخلص *Argania spinosa* وزيت *Thymoquinone* و *Nigella sativa* و مستخلص *lyophiled* ضد ارتفاع الهوموسيستين في الدم.

العلاج بالفيتامينات، يمكن أيضًا اعتباره علاج مضاد للأكسدة والذي تم الحصول عليه من المواد الكيميائية الموجودة بالنباتات ، وهو مصدر لمضادات الأكسدة الطبيعية للحماية من أضرار جذور الأكسجين الحرة المنتجة

بواسطة ارتفاع الهوموسيستيين (Benmebarek *et al*., 2013).

أثبتت الدراسات الحديثة أن مضادات الأكسدة يمكن أن تخفض مستويات الهوموسيستيين بشكل جد فعال (Çelik *et al*., 2017) ، أظهرت دراسة (Labed *et al*., 2016) أن مستخلص أسيتات الإيثيل من *A. armatus* يحتوي على فلافونويدات، وقد أظهر هذا المستخلص نشاط مضاد للأكسدة عالي في فحوصات DPPH، ABTS و CUPRAC.

تعتبر المركبات الفينولية وكذلك الفلافونويدات كمضادات أكسدة و التي لها فوائد صحية عديدة منها علاج العديد من الأمراض والوقاية منها (Tungmunnithum *et al*., 2018)، و منه نستنتج أن التأثير الوقائي لمستخلص *A. armatus* ضد ارتفاع الهوموسيستيين راجع إلى مركب الفلافونويد المعروف كمضاد أكسدة قوي.

بالإضافة إلى ذلك، نتائجنا بينت أن تناول مستخلص نبتة *A.armatus* مع الميثيونين تسبب في انخفاض مستويات الدهون في الدم. أكدت دراسة (Seo *et al*., 2010) أن تناول كميات كبيرة من مضادات الأكسدة يعتبر كعامل وقائي ضد تصلب الشرايين، وربما يكون له تأثير إيجابي على أكسدة دهون LDL عند أخذه مع مستويات عالية من الهوموسيستيين و دهونLDL . تشير الدراسات الحديثة إلى أن بعض أنواع البوليفينول له تأثير مفيد على خلل دهون الدم في الإنسان و الحيوانات(Mulvihill and Huff, 2010).

علاوة على ذلك، أكدت نتائجنا أن إعطاء و تناول مستخلص نبتة *A. armanus* بالاشتراك مع الميثيونين أدى إلى ارتفاع مستوى مضادات الأكسدة الإنزيمية GSH و CAT، و تتفق نتائجنا مع الأبحاث التجريبية السابقة بواسطة

(Aklil *et al*., 2017; El-Saleh *et al*., 2004; Çelik *et al*., 2017)،حيث أظهروا أن مستخلص *Argania spinosa*، وزيت الثيموكينون و زيت الحبة السوداء يمكن أن يكون له تأثير وقائي ضد الإجهاد التأكسدي الناتج عن فرط الهوموسيستيين في دم الفئران.

ممكن أن ترتبط إحدى الآليات المحتملة للتأثير الوقائي ل Quercetin بخصائصه المضادة للأكسدة، والتي تزيد من أنشطة مضادات الأكسدة الإنزيمية مثل SOD، CAT وGSH ، و مضادات الأكسدة غير الأنزيمية في

البلازما (Meng *et al*., 2013).

من جهة أخرى بينت نتائجنا أن تناول مستخلص نبتة *A.armatus* مع الميثيونين أدى إلى تحسين التغيرات النسيجية، وتصحيح التشوهات الهيكلية التي لوحظت في الشريان الأورطي ، القلب وأنسجة الكبد و ذلك عند تناول جرعات عالية من الميثيونين.

نتائجنا تتوافق مع دراسة (Aklil *et al*., 2017; Benmebarek *et al*., 2013) التي أكدت أن فرط الهوموسيستيين الناجم عن اتباع نظام غذائي عالي الميثيونين يمكن أن يتلف الشريان الأورطي وأنسجة القلب ، وأن تغذية هذه الحيوانات ببذور *Argania spinosa* المجففة، ومستخلص *Stachys mialhesi* يصحح هذه التشوهات.

كما أشارت نتائج (Yalçınkaya *et al*., 2009) أن taurine له تأثيرات وقائية على السمية التي يسببها فرط الهوموسيستيين، عن طريق تقليل كل من الإجهاد التأكسدي، الموت المبرمج الخلوي والنخر في الكبد.

قد تكون التأثيرات الوقائية المحتملة ضد أمراض القلب ناتجة عن قدرة بعض البوليفينولات على منع أكسدة دهون LDL و بذلك تمنع حدوث تصلب الشرايين (Duthie *et al*., 2000)، كما أفادت الدراسة التجريبية التي أجراها (Benmebarek *et al*., 2013) أن علاج فرط الهوموسيستيين بمستخلص نبتة *Stachys mialhesi* المضاد للأكسدة والمضاد للالتهابات يمنع إصابة البطانة وتلف الكبد.

تم التأكد من احتواء مستخلص *A.armatus* على مادة الفلافونويد التي تشير إلى نشاط عالي مضاد للأكسدة (Labed *et al*., 2016)، وترجع الخاصية المضادة للأكسدة للفلافونويدات بدرجة عالية إلى استبدال جزيئة OH.

علاوة على ذلك، فإن تثبيط الوظائف الأنزيمية بخلاف إنزيمات Oxidases ، مثل تثبيط إنزيم lipoxygenase وبالتالي حماية تكوين الليكوترينات leukotrienes، قد يشارك أيضًا في الخصائص الوقائية للخلية والأنسجة بواسطة الفلافونويدات (De Groot and Ruin, 1998).

2- تقييم النشاط المضاد للأكسدة للكسر البوتانولي لنبتة *A. armatus*

أظهرت نتائج التقييم خارج العضوية للنشاط المضاد للأكسدة للكسر البوتانولي لنبتة *A.armatus* باستعمال

أربع طرق: اختبار القدرة الكلية المضادة للأكسدة، اختبارالقدرة الإختزالية، اختبار القدرة على إقتناص الجذور الحرة (DPPH˙)، اختبارالقدرة على استخلاص المعادن، أظهرت نشاطًا و قدرة منخفضة في الفعل المضاد للأكسدة الكلية، القدرة الإختزالية، القدرة على إقتناص الجذور الحرة، استخلاص المعادن لهذا المستخلص.

هذه النتيجة يمكن مقارنتها بالدراسة التي أجراها (Labed *et al*., 2016) الذي أكد أن النشاط المضاد للأكسدة لكسر(AcOEt) أعلى من نشاط كسر البوتانول المنخفض من مستخلص نبتة *A. armatus* ، لأن الكسر البوتانولي لهذه النبتة يحتوي على 3 وضعيات للفلافونويد الجليكوزي 3-position glycosylated flavonoids، مما يقلل بشكل كبير من نشاط مضادات الأكسدة.

3 - التأثير الوقائي للكسر البوتانولي لنبتة *A. armatus* على النمو والنشاط التكاثري لبعض الخلايا السرطانية مثل خلايا HeLa وخلايا C 6

يشكل السرطان عبئا كبيرا على الصحة العامة في البلدان المتقدمة النمو والبلدان النامية على السواء، و يعرف النشاط المضاد للسرطان أنه تأثير العوامل الطبيعية، الصناعية أو البيولوجية والكيميائية لقمع أو منع التطور للخلايا المسرطنة. و يتم استخدام العديد من العوامل الصناعية لعلاج المرض ولكن لها سمية وبالتالي فإن البحث مستمر للتحقيق في عوامل العلاج الكيميائي المشتقة من النبات (Shaikh *et al*., 2016).

تتم دراسة الأنشطة المضادة للتكاثر على نطاق واسع للنباتات الطبية والمصادر الشائعة الأخرى (Demirtas *et al*., 2009).

تم اختبار الخصائص المضادة للسرطان لمستخلص الكسر البوتانولي لنبتة *A. armatus* ضد خلايا HeLa (سرطان عنق الرحم البشري)، وخلايا C6 (ورم دماغ الجرذان)، باستخدام جهاز BrdU ELISA cell proliferation assay ومقارنتها بعنصرالتحكم الإيجابي في (5-FU-5). و قد أكدت نتائجنا أن استعمال تراكيز مختلفة من هذا المسخلص يؤدي إلى تثبيط النمو والنشاط التكاثري لبعض الخلايا السرطانية مثل خلايا HeLa وخلايا C6 ، بالإضافة إلى ذلك، تم تقييم مستخلص الكسر البوتانولي لنبتة *A. armatus* أنه يحتوي على أنشطة عالية مضادة للتكاثر ضد خلايا C6 مقارنة بخلايا HeLa عند التركيز100 ميكروغرام/مل.

كما ذكرنا سابقا، أعطت الدراسة الكيميائية النباتية لمستخلص الكسر البوتانولي لنبتة *A. armatus* أربعة

مركبات فلافونويد (Labed *et al.*, 2016). حيث تعتبر مركبات الفلافونويد مركبات نباتية نشطة بيولوجيًا ذات أهمية كبيرة في التغذية والصيدلة، نظرًا لخصائصها القوية كمضادات للأكسدة، مضادة للالتهابات، مضادة للجراثيم، مضادة للفطريات ومضادة للأورام (Fernandez *et al.*, 2021) .

كما أظهرت الدراسات التي أجريت فخارج العضوية وفي الجسم الحي أن بعض مركبات الفلافونويد تعدل التمثيل الغذائي وتساعد على التخلص من المواد المسرطنة ويمكن أن تساهم في الوقاية من السرطان (Senderowicz, 2001; Carroll *et al.*, 1998). و قد ثبت أن مركبات الفلافونويد تحفز موت الخلايا المبرمج في بعض خطوط الخلايا السرطانية ، بينما تحافظ على الخلايا الطبيعية، بسبب أنها مركبات فعالة بشكل خاص في تثبيط أوكسيداز الكزانثين Xanthine oxidase ، وبالتالي تمنع تكاثر الخلايا السرطانية (Ren *et al.*, 2003).

الخاتمة

الخاتمة

لا تزال أمراض القلب والأوعية الدموية هي السبب الرئيسي للوفيات في جميع أنحاء العالم، وقد وُصفت زيادة مستويات الهوموسيستيين في الدم بأنها عوامل خطر لأمراض القلب والأوعية الدموية .

في هذه الدراسة، قمنا بإثبات هذه الحقيقة، حيث قمنا أولا باستحداث حالة فرط الهوموسيستيين لدى الفئران التي أعطيناها جرعة عالية من الميثيونين (400 مغ/ كغ) خلال 21 يومًا، أيضا قمنا بتقييم التأثير الوقائي لمستخلص (AcOEt) لنبتة *A. armatus* ضد التشوهات الأيضية والنسيجية التي تحدث في الفئران التي تعاني من حالة فرط الهوموسيستيين و التي عولجت بـالميثيونين L-methionine.

من ناحية أخرى ، قمنا بتقييم النشاط المضاد للأكسدة و تقييم النشاط المضاد للتكاثر للمستخلص البوتانولي (n-BuOH) لنبتة *A. armatus* خارج العضوية.

- نتائجنا بينت أن استهلاك نظام غذائي عالي الميثيونين (400 مغ/ كغ) خلال 21 يومًا أدى إلى زيادة معتبرة في الهوموسيستيين في البلازما. علاوة على ذلك ، أدى إلى زيادة في تركيزات الدهون و انخفاض في HDL-c، الجلوتاثيون (GSH) و الكاتلاز (CAT).
- بينت الفحوصات النسيجية في الفئران التي تعاني من فرط الهوموسستئين في الدم ظهور تغيرات نسيجية في الشريان الأورطي، القلب و الكبد، وقد لوحظ ذلك من خلال التحلل والنخر في بنية الألياف العضلية، وتسلل الخلايا أحادية النواة بين الخلايا العضلية في أنسجة القلب.
- بينما أظهرت هذه الفحوصات لشريان الأبهر تقشر الخلايا الطلائية، ولاحظنا أيضًا تشكل الفجوات، وتكوين الخلايا الرغوية والأنوية البيضاوية.
- بالإضافة إلى ذلك، فإن الملاحظة المجهرية لنسيج الكبد في الفئران التي عولجت بـكميات عالية من الميثيونين كشفت عن تلف الكبد المرضي الشديد الذي يتميز بالنخر، وتغير في شكل نوى الخلايا الكبدية وظهور حالة التشحم الكبدي.

- في حين أن إعطاء مستخلص *A. armatus* ((100مغ/ كغ) مع الميثيونين (400 مغ/ كغ)، تسبب في انخفاض في تركيز الهوموسيستيين في الدم و أيضا انخفاض في مستويات الدهون مع زيادة في نشاط مضادات الاكسدة الإنزيمية الجلوثاتيون و الكاتالاز GSH و CAT، أيضا أدى تناول هذا المستخلص إلى تحسين التغيرات

والتشوهات النسيجية التي أحدثها ارتفاع الهوموسيستيين على مستوى أنسجة كل من القلب، شريان الاورطى والكبد.

- أظهرت نتائج التقييم خارج العضوية للنشاط المضاد للأكسدة للكسر البوتانولي لنبتة *A.armatus* باستعمال أربع طرق ، نشاطًا و قدرة منخفضة في الفعل المضاد للأكسدة الكلية، القدرة الإختزالية، القدرة على إقتناص الجذور الحرة و استخلاص المعادن لهذا المستخلص.
- بينت نتائج التقييم خارج العضوية للنشاط المضاد للتكاثر لمستخلص للكسر البوتانولي لنبتة *A armatus* ضد خلايا HeLa (سرطان عنق الرحم البشري) وخلايا C6 (ورم دماغ الجرذان)، أن استعمال تراكيز مختلفة من هذا المسخلص يؤدي إلى تثبيط النمو والنشاط التكاثري للخلايا السرطانية مثل خلايا HeLa وخلايا C6 ، بالإضافة إلى ذلك، تم تقييم مستخلص الكسر البوتانولي لنبتة *A. armatus* أنه يحتوي على أنشطة عالية مضادة للتكاثر ضد خلايا C6 مقارنة بخلايا HeLa في التركيز 100 ميكروغرام/مل.

أظهرت النتائج المتحصل عليها في هذه الدراسة أن نبات *A. armatus* يمكن اعتباره مصدرًا طبيعيًا للوقاية من أمراض القلب والأوعية الدموية والسرطان.

وبناء على النتائج المتحصل عليها في هذه الدراسة، نطمح في المستقبل القيام و البحث في مجموعة من المواضيع وهي على التوالي:

- ✓ إستخلاص وتحديد الجزيئات النشطة بيولوجيا الواردة في المستخلص النباتي.
- ✓ تقييم نشاط مضادات أكسدة إنزيمية أخرى مثل Superoxide dimustase.
- ✓ دراسة التعبير الجيني للإنزيمات المضادة للأكسدة التي يحتويها المستخلص النباتي، و إظهار دورها في علاجات القلب والأوعية الدموية .

الملخص

يرتبط فرط الهوموسيستين في الدم، الذي يتميز بزيادة مستوى الهوموسيستين في البلازما، بأمراض القلب والأوعية الدموية، تصلب الشرايين،الإصابات الكبدية، والتمثيل الغذائي غير الطبيعي للدهون، كما يعتبر أيضًا عامل خطر للإصابة بالسرطان. أكدت العديد من الدراسات انخفاض مستوى الهوموسيستين في البلازما عن طريق العديد من المركبات المضادة للأكسدة الفينولية والمستخلصات النباتية. أظهرت الدراسات أن بعض مركبات الفلافونويد يمكن أن تساهم في الوقاية من السرطان وهي فعالة بشكل خاص في منع تكاثر الخلايا السرطانية.

لقد أجرينا هذا البحث لتقييم التأثير الوقائي لمستخلص الإثيل أسيتات (AcOEt) لنبتة *A. armatus* على أمراض القلب والأوعية الدموية بعد إحداث حالة فرط الهوموسيستيين، و أيضا تقييم النشاط المضاد للأكسدة والنشاط المضاد للتكاثر للمستخلص البوتانولي (n-BuOH) لنبتة *A. armatus* في العضوية.

أدى استهلاك نظام غذائي عالي من الميثيونين ﴿400 ملغ/كلغ﴾ خلال 21 يومًا إلى إلى زيادة معتبرة في الهوموسيستيين في البلازما. علاوة على ذلك، أدى إلى زيادة في تركيزات الدهون، وانخفاض في HDL-c، الجلوتاثيون (GSH) و الكاتلاز (CAT). بالإضافة إلى ظهور تغيرات و تشوهات نسيجية في الشريان الأورطي، القلب و الكبد. في حين أن إعطاء مستخلص نبتة *A. armatus* مع الميثيونين أدى إلى انخفاض في تركيز كل من الهوموسيستيين، الدهون، وزيادة في نشاط الجلوتاثيون و الكاتالاز، مع تحسين التغيرات و التشوهات النسيجية .كما بينت نتائجنا أن تقييم النشاط المضاد للأكسدة خارج العضوية لمستخلص الكسر البوتانولي لنبتة *A. armatus* بأربع طرق، أعطى نشاطا منخفضا. بينما بينت نتائج التقييم في المختبر للنشاط المضاد للتكاثر لمستخلص الكسر البوتانولي لنبتة *A. armatus* ضد خلايا HeLa سرطان عنق الرحم البشري وخلايا C6 ورم دماغ الجرذان، أن استعمال تراكيز مختلفة من هذا المسخلص يؤدي إلى تثبيط النمو والنشاط التكاثري للخلايا السرطانية مثل خلايا HeLa وخلايا C6، بالإضافة إلى ذلك، تم تقييم مستخلص الكسر البوتانولي لنبتة *A. armatus* أنه يحتوي على أنشطة عالية مضادة للتكاثر ضد خلايا C6 مقارنة بخلايا HeLa عند التركيز100 ميكروغرام/مل.

أظهرت النتائج المتحصل عليها في هذه الدراسة أن نبات *A. armatus* يمكن اعتباره مصدرًا طبيعيًا للوقاية من أمراض القلب والأوعية الدموية والسرطان.

الكلمات المفتاحية: فرط الهوموسيستين في الدم، أمراض القلب والأوعية الدموية، *Astragalus armatus*، مضادات الأكسدة الإنزيمية، النشاط المضاد للأكسدة، النشاط المضاد للتكاثر، خلايا HeLa وخلايا C6 .

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THE PROTECTIVE EFFECT OF *ASTRAGALUS ARMATUS* ON CARDIOVASCULAR DISEASES INDUCED BY HYPERHOMOCYSTEINEMIA IN MICE

Ibtissam Baghriche[1,2,3], Sakina Zerizer[1,2*], Zahia Kabouche[1], Assia khalfallah[1]

[1]Université des frères Mentouri Constantine 1, Laboratoire d'Obtention de Substances Thérapeutiques (LOST), 25000 Constantine, Algeria;*

[2]Université des frères Mentouri-Constantine, Département de Biologie Animale, Algeria;*

[3]Assia Djebar Teachers Training School of Constantine, Department of Natural Sciences, Ville Universitaire Ali Mendjeli, 25000 Constantine, Algeria;

[*]Corresponding Author Sakina Zerizer, e-mail: zerizer.sakina@umc.edu.dz;

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ABSTRACT

Our research aims to determine the protective effect of the *Astragalus armatus* extract on plasma homocysteine (Hcy) rate, lipids, antioxidant enzymes and histological abnormalities of aorta and heart, in hyperhomocysteinemia (HHcy) induced by high-methionine died in mice, which is an independent risk factor for cardiovascular diseases. Twenty eight adult male *Mus Musculus* mice were divided into four groups, the control group (F) was fed with white bread, group (M) was fed with L-methionine, group (PM) was fed with L-methionine plus *A. armatus* extract, and the group (P) was treated with *A. armatus* extract. After 21 days of treatments, Hcy concentration, lipid parameters, hepatic antioxidant status and histological sections of aorta and heart were determined. Consumption of high methionine diet resulted in a significant increase in plasma Hcy. Furthermore, we detected an increase in lipid parameters concentrations, and a decrease in HDL-c, glutathione reduced (GSH) and catalase (CAT) activities. These results are associated with the appearance of pathological alterations in the aorta and the heart organs. While the administration of *A. armatus* extract with L-methionine caused: a decrease in Hcy concentration and lipid parameters, an increase in GSH and CAT activities, and an improvement in histological changes. Our data showed that *A. armatus* extract is effective in: decreasing plasma Hcy levels and lipid parameters, reducing oxidative stress by increasing antioxidant status and protecting aorta and heart tissues in mice fed a diet rich in L-methionine.

Keywords: Hyperhomocysteinemia, *Astragalus armatus*, lipids status, Glutathione, Catalase.

INTRODUCTION

Homocysteine is a sulfur amino acid. Its metabolism involves two remethylation pathways to methionine, which needs folate and vitamin B12, and transsulfuration to cystathionine, which needs vitamin B6 (Selhub, 1999). Hcy can cause widespread hazards to the human body in case of HHcy; defined by the increase in the Hcy level in the plasma (Zhang *et al.*, 2005). HHcy is related with cardiovascular disease, atherosclerosis and reactive oxygen (Mendes *et al.*, 2014), hepatic lesions and abnormal lipid metabolism (Latour *et al.*, 2015).

Cellular defense against reactive oxygen species (ROS) are conducted through intracellular systems such as antioxidant enzymes or decreasing agents such as vitamin C (Filip *et al.*, 2010).

Many studies have confirmed the discount of plasma Hcy level by several phenolic antioxidant compounds and

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plant extracts like coffee, catechin, red wine phenolic and chlorogenic acid (Nygard *et al*., 1997; Noll *et al*., 2013; Noll *et al*., 2011; Kim *et al*., 2012), in order to improve and enrich these treatments, we have focused our study on herbal medicine. Until recently, plants were an important source of novel pharmacologically active compounds so many blockbuster drugs are directly or indirectly derived from plants (Veeresham, 2012). *Astragalus armatus* Willd (Fabaceae) locally known as ''ketad'' is an endemic species in Algeria. Furthermore, the aerial parts of *A. armatus* contain several bioactive compounds including flavonol glycosides and triglycoside such as narcissin, nikotiflorin, and mauritianin (Khalfallah *et al*., 2014). The flavonoid Isorhamnetin was obtained from Ethyl acetate extract of *A. armatus* which indicated the highest antioxidant activity in DPPH, ABTS and CUPRAC assays (Labed *et al*., 2016). We have conducted this research to confirm and evaluate the protective effect of the *A. armatus* plant extract on the plasma Hcy rate, lipid status, antioxidant status and histological abnormalities of aorta and heart, caused by the high dose of L-methionine induced in mice.

MATERIAL AND METHODS

Material

Plant Material

Aerial parts of *A. armatus* were collected from Bekira-Constantine (Eastern Algerian) in May 2007. The voucher specimen was kept in the Herbarium of the Faculty of Sciences (University of Constantine-1) under the number LOST. Aa.05.07. (Khalfallah *et al*., 2014). In our study, the extract was tested in the ethyl acetate phase.

Chemicals Material

The concentration of homocysteine in plasma was determined by the Immulit homocysteine Kit (Siemens, Finland), other used chemicals were obtained from the chemical company Sigma.

Animal Material

The research was performed on twenty eight healthy adult male *Mus Musculus* mice, 2.5 to 3 months old, weighing between 30 and 35 g. All animals were obtained from the Central Pharmacy of Algeria and housed in plastic cages with free access to water and diets for 21 days with experimental diet control. Mice were divided into four groups of similar mean body weights and conducted under standard laboratory conditions of humidity, temperature and light. They received tap water and a standard laboratory diet purchased from EL REGHAIA feed Co. (Algiers, Algeria).

Methods

Experimental design

After an acclimatization period of one week, the pubertal mice were randomly assigned into 4 groups (7 mice per group): The control group (F) was fed with white flour (0.50 mg/mouse), the group (M) was fed with L-methionine (400 mg/kg/day), the group (MP) received L-methionine (400 mg/kg/day) plus *A. armatus* extract (100 mg/kg/day), finally the positive control group (P) was treated with *A. armatus* extract (100 mg/kg/day). L-mehionine and *A. armatus* extract were orally administered to mice with white flour (0.50 mg/mouse) and given free access to food and water. After 21 days of feeding, animals have fasted overnight and before sacrificing animals, blood samples were taken from the retro-orbital plexus into EDTA tubes and centrifuged, the plasma was stored at -20˚C until biochemical analysis. The aorta and heart were immediately removed and washed with PBS saline, a fraction of each animal's liver was used to determine biochemical parameters while other fractions of aorta and heart of each animal were used for histological examination. The experiments were carried out in strict compliance with ethical principles set out by the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on the Animal).

Biochemical analysis

Measurement of plasma Hcy levels, lipids status

The determination of the plasma Hcy and lipids status was carried out at the IBN SINA medical analysis laboratory in Constantine.

Preparation of homogenate

For the determination of oxidative stress parameters of GSH and CAT activities, the liver (0.5 g) was homogenized in 2 ml of TBS solution; the homogenates were centrifuged at 9000 ×g for 15 min at 4˚C. After that, the pellet was discarded and the supernatant was subjected to biochemical assays.

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Protein determination

Protein concentrations were determined by the Bradford method (Bradford, 1976). The absorption was read at 595 nm on a spectrophotometer and the results are expressed in mg/ml protein.

Determination of glutathione reduced

The concentration of the glutathione reduced in the liver was determined according by the method of Weckbecker and Cory (Weckbercker and Cory, 1988), The GSH in the liver was measured spectrophotometrically by using 5, 5′-dithiobis-(2 nitrobenzoic acids) (DTNB) as a coloring reagent, GSH levels were spectrophotometrically determined at 412 nm.

Determination of catalase

Catalase activity was assayed in mouse liver according to the method of Aebi (Aebi, 1974). This is carried by measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100 and 10 mM potassium phosphate buffer, Ph=7. The CAT unit is defined as 1 m mol of hydrogen peroxide consumed per minute and the specific activity is reported as units/mg of protein (Stefanello *et al*., 2009).

Histological procedure

After the blood samples collection, the animals were sacrificed and organs designed for morphological analysis (aorta, heart) were quickly removed, rinsed with saline solution 0.9%, and fixed in formalin 10%. The processed tissues were embedded in paraffin, sectioned at 5 µm thickness, and stained using the hematoxylin-eosin staining method (Aklil *et al*., 2017).

Statistical Analysis

The biochemical analysis (Hcy), lipids status, GSH and CAT assay are presented as (mean±SEM). The data were compared between groups using one-way ANOVA test and Tukey's multiple comparison tests (SPSS version 20). Differences were considered statistically significant (*) if $P < 0.05$, and highly significant (**) if $P < 0.01$.

RESULTS

Plasma Hcy concentration

After 21 days, group (M) plasma Hcy reached higher levels where the mean was (13.48±3.71 µmol/l). This increase is not significant when comparing it to the control group (F) (8.68±1.89 µmol/l) for which $P>0.05$. Conversely, its increase is significant compared to the positive control group (P) (7.22±1.365 µmol/l) where $P=0.014$. The Hcy concentration in the (PM) group (treated with *A. armatus* extract plus L-methionine) is (9.49±3.53 µmol/L); decreased compared to group (M) mice treated with 400 (mg/kg/day) of L-methionine. The significant increase in Hcy by the high dose of L-methionine was corrected by the plant extract of *A. armatus* (Figure1).

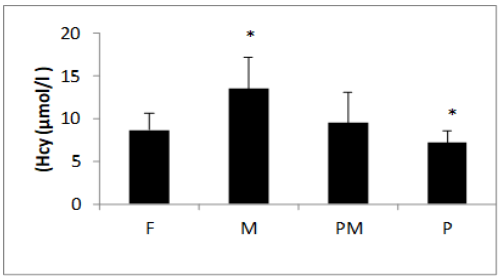

Figure 1. The interaction of L-methionine and *A. armatus* extract on Hcy in mice during 21 days of treatment,
(F) Control group, (M) Treated with L-methionine, (PM) Treated with L-methionine and A. armatus extract,

*(P) Treated with A. armatus extract. Values are the means±SEM (n); *p<0.05.*

Lipids status

Data showed that after 21 days, the levels of Tch increased not significantly in the group (M) (1.074±0.13 g/l) when it is compared to the control group (F) (0.99±0.31 g/l). In addition, they lowered in the (PM) group (0.938±0.08 g/l) but increased in the (P) group (1.19±0.20 g/l).

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However, LDL-c levels increased significantly in group (M) (0.65±0.20 g/l) where P = 0.016 and non-significantly decreased in both group (P) (0.53±0.33 g/l) and group (PM) (0.37±0.16 g/l), compared to the control group (F) (0.18 ± 0.07 g/l).
In the other hand, HDL-c levels decreased non-significantly in group (M) (0.75±0.185 g/l) compared to groups (F) (0.80±0.09 g/l), (P) (0 .66±0.464 g/l) and (PM) (0.66±0.435 g/l).
In addition, TG levels increased non-significantly in group (M) (1.19±0.348 g/l) compared to group (F) (0.92±0.36 g/l) and decreased in both (PM) (0.78±0.060 g/l) and (P) (0.90±0.135g/l) groups (Figure2).

Antioxidant enzyme activities

The values obtained during this study showed a high and significant difference between the groups in terms of mean concentration of GSH (P = 0.005) as well as for CAT showed a significant difference between the groups (P = 0.042). In group (M), GSH values (10.63±7.15 n mol/mg protein) decreased sharply and significantly compared to the positive control group (P) (26.10±7.53 n mol/mg protein) with P = 0.002. In addition, the GSH level increased non-significantly in the (PM) group (17.39±4.08 n mol/mg protein).
However, GSH values were significantly elevated in group (P) (26.10±7.53 n mol/mg protein) compared to control group (F) (16.67±2.18 n mol/mg protein) with P=0.049.
CAT concentration decreased significantly in the group (M) (21.74±19.86 m mol/mg protein) compared to group (P) (53.34±11.51 m mol/mg protein) with P = 0.028. Nevertheless, CAT concentration increased non-significantly in the (PM) group (42.28±19.93 m mol/mg protein) and in the (F) control group (36.94±18 .55 m mol/mg of protein) (Figure3).

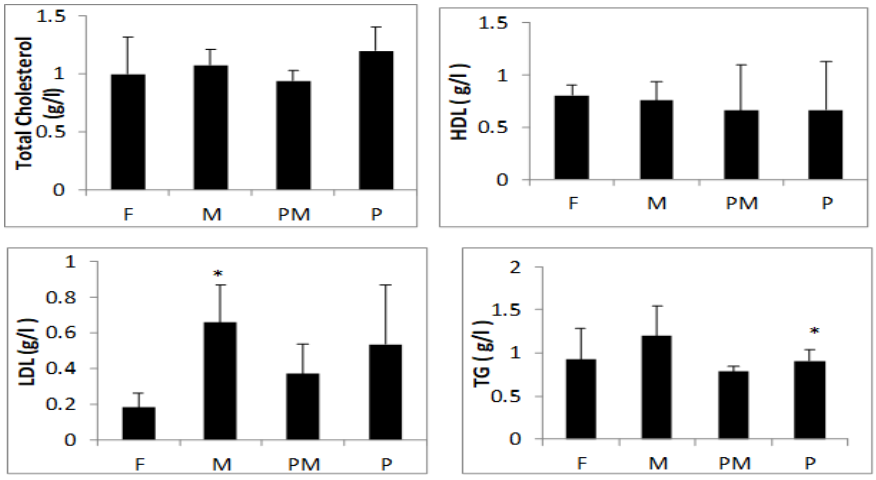

Figure 2. The interaction of L-methionine and *A. armatus* extract on lipids status (Tch, LDL-c, HDL-c, TG) in mice, during 21 days of treatment,

*(F) Control group, (M) Treated with L-methionine, (PM) Treated with L-methionine and A. armatus extract, (P) Treated with A. armatus extract. Values are the means±SEM (n); *p<0.05.*

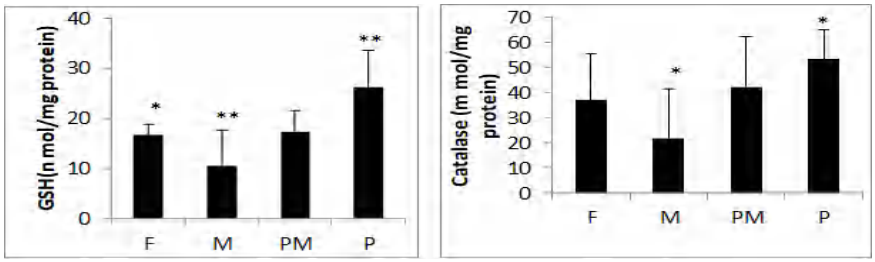

Figure 3. The interaction of L-methionine and *A. armatus* extract on glutathione reduced values (GSH) and catalase activity (CAT) in mice, during 21 days of treatment,

*(F) Control group, (M) Treated with L-methionine, (PM) Treated with L-methionine and A. armatus extract, (P) Treated with A. armatus extract. Values are the means±SEM (n); (*p<0.05, **p<0.01).*

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Histological investigations

For group (M) which received L-methionine (400mg/kg/day), light microscopy of the heart showed lysis and necrosis in the muscle fiber structure and mononuclear cell infiltration between the muscle cells (Figure 4-C, 4-D). Thisis not the case for group (F) which presented a normal tissue structure (Figure 4-A, 4-B). Same results were also observed in PM and P groups (Figure 4-E, 4-F).

Microscopic observation of aortic intima in the methionine group (M) showed desquamation of endothelial cells, we also observed in the media lysis, gaps, formation of foam cells and oval nuclei (Figure 5- B, 5-C, 5-D). However, in the control group (F), histopathological examination showed a normal structure, the aortic section have intact endothelium and spindle shaped nuclei, (Figure5-A,). For the MP and P groups, the observation presents an intact morphology (Figure 5-E, 5-F).

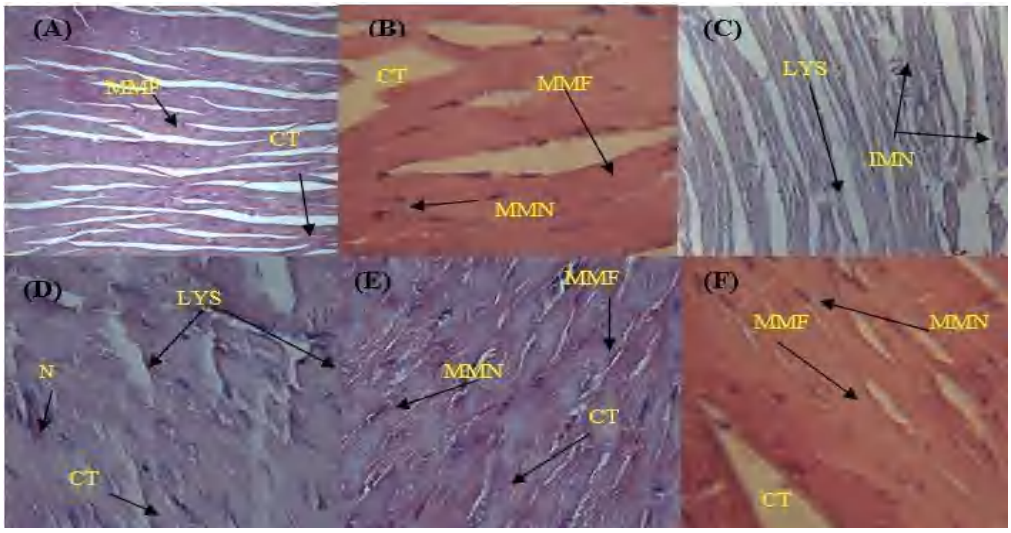

Figure 4. Histological section of the heart, during 21 days of treatment,

(A and B) Control group, (C and D) Treated with L-methionine, (E) Treated with L-methionine and A. armatus extract, (F) Treated with A. armatus extract. Application hematoxylin-eosin staining, (A, C) x100, (B, D, E, F) x400. MMN: Muscular myocard nuclei, MMF: Muscular myocardfibers, CT: Connective tissue. N: Necrosis, LYS: Lysis, IMN: Infiltration of mononuclear.

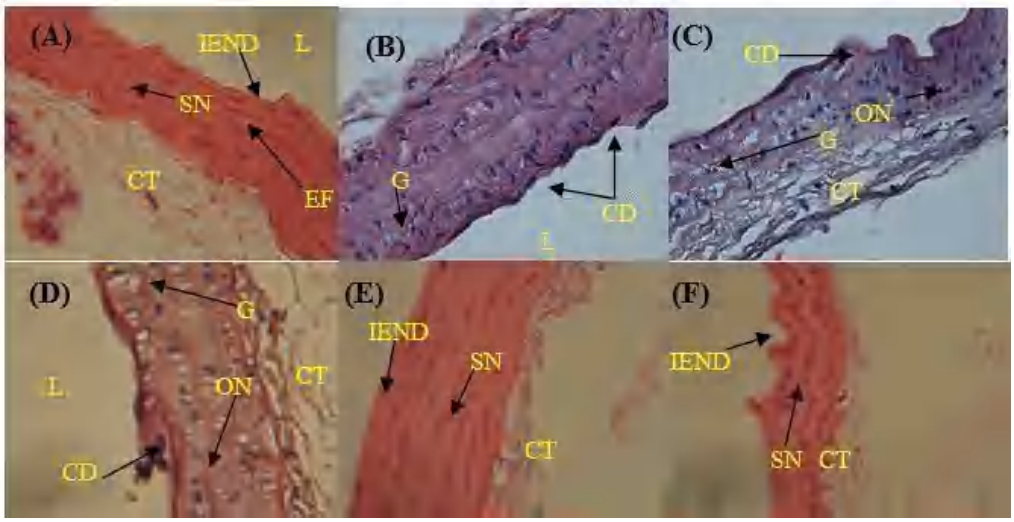

Figure 5. Histological section of the aorta, during 21 days of treatment,

(A) control group, (B, C and D) treated with L-methionine, (E) treated with L-methionine and A. armatus extract, (F) treated with A. armatus extract. Application hematoxylin-eosin staining, (A, F) x100, (B, C, D, E) x400. L: Lumen, IEND: Intact Endothelium, CT: Connective tissue, EF:

Elastic Fibers, SN: Spindle nuclei, ON: Oval Nuclei, FC: Foam cells, Lys: Lysis, G: Gaps, CD: Cellular Desquamation.

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Discussion

Hyperhomocysteinemia, characterized by high plasma homocysteine levels, is an important risk factor for cardiovascular diseases (Boyacioglu *et al*., 2014), and is thought to produce endogenous oxidative stress and causes many cellular damages (Derouiche *et al*., 2014). An important role in development of hyperhomocysteinemia is also played by deficiency of folic acid, vitamins B6 and B12 (Domagala *et al*., 1997), however, may be prevented by polyphenols, potent antioxidant compounds with anti atherogenic properties (Noll *et al*., 2011). The use of vitamins to modulate homocysteine metabolism substantially lowers the risk by reducing plasma homocysteine levels (Sim *et al*., 2016).

Our current results showed that a methionine enriched diet in mice for 21 days caused a significant increase in Hcy concentration, an increase in the concentrations of Tch, LDL-c and TG, decrease in HDL-c, GSH and CAT, these results are associated with the appearance structural alterations of organs aorta and heart.

Obtained results are in agreement with the previous experimental study of (Boyacioglu *et al*., 2014; Derouiche *et al*.,2014) which have shown that Consumption of high methionine diet induced a significant increase of plasma Hcy. Hyperhomocysteinemia leads to diverse clinical manifestations, the pathogenicity of homocysteine is believed to be due to its ability to produce oxidative stress (Noll *et al*., 2011)

Furthermore, we detected an increase in the concentrations of Tch, LDL-c and TG, as well as a decrease in HDL-c, GSH and CAT following the oral administration of L-methionine, our result demonstrated a positive correlation between Hcy and the lipid profile (Tch, LDL-c and TG) as well as a negative correlation with HDL-c. This is in agreement with the previous experimental study by (Obeid *et al*., 2009; Momin *et al*., 2017; Shaker *et al*., 2013).

An association between hyperlipidemia and HHcy has been suggested However, results from (Liao *et al*., 2006) indicated that HHcy inhibits reverse cholesterol transport by reducing circulating HDL-c. This is done through inhibiting apoA-I protein synthesis and enhancing HDL-c clearance. Also the study of (Obeid *et al*., 2009) demonstrate that the effect of Hcy on HDL-cholesterol is probably related to the inhibition of enzymes or molecules participating in the HDL-particle assembly.

The increase in LDL seems to be linked to their peroxidation by free radicals generated by HHcy because this peroxidation inhibits the recognition of oxidized LDL by their native receptors (Laporte, 2000). The work of (Werstuck *et al*., 2001) proved that accumulation of hepatic cholesterol and triglycerides in HHcy is associated to an increase in the biosynthesis and absorption rather than a reduction in the hepatic export of VLDL.

Furthermore, our data showed a decrease in GSH and CAT activities as a biochemical marker of oxidative stress in mice given a high dose of L-methionine, Hcy reduced enzymatic antioxidant potential in tissues. Moreover, oxygen radicals may play an important role in this specific HHcy model (Boyacioglu *et al*., 2014), long-term methionine treatment promotes oxidative stress as it decreases non-enzymatic antioxidant defenses, increases lipid peroxidation and protein carbonylation, alters activity of antioxidant enzymes, and changes in serum biochemical parameters (Stefanello *et al*., 2009 and Matté *et al*., 2009).In addition to increasing H_2O_2 generation, Hcy decreases the cell's ability to detoxify H_2O_2 by impairing intracellular antioxidant enzymes, specifically the intracellular isoform of glutathione peroxidase (GPx) (Upchurch *et al*., 1997).

Catalase, an antioxidant enzyme responsible for the hydrogen peroxide degradation, is protective in many diseases. Homocysteine inhibition may increase the levels of hydrogen peroxide and play a role in disease pathology (Nathaniel, 2008).

Histopathological examinations in hyperhomocysteinemic mice, showed an appearance of structural alterations on the aorta and heart tissue damages, This was observed through lysis and necrosis in the muscle fiber structure, and mononuclear cell infiltration between the muscle cells in the heart tissue, the aortic intima showed desquamation of endothelial cells, we also remarked in the media lysis, gaps, formation of foam cells and oval nuclei, our results are in agreement with (Aklil *et al*., 2017 and Benmebarek *et al*., 2013) which reported that The oral administration of methionine exerted an angiotoxic activity on the aorta and a toxic effect on the heart.

HHcy acts via oxidative stress to promote myocardial fibrosis and dysfunction (Joseph *et al*., 2008). Increased oxidant stress appears to play a pathophysiological role in the deleterious endothelial predispose affected vessels to the subsequent development of atherosclerosis predispose affected vessels to the subsequent development of atherosclerosis effects of homocysteine (Kanani *et al*., 1999). An association between the presence of abdominal aortic aneurysm and high homocysteine plasma levels has been mentioned, homocysteine plays an important role in development of aortic dissection (Takagi *et al*., 2005), the results of (Lamda *et al*., 2014) show that elevated plasma homocysteine increase cholesterol synthesis, exerts an angiotoxic action direct to aorta through loss of endothelium, degeneration partly with dissolution of media cells.

Further, we found that the treatment with the *A. armatus* extract was effective in protection against HHcy in mice fed a diet rich in L-methionine by decreasing plasma Hcy levels, decreasing lipid parameters and reducing oxidative stress by increasing the concentrations of GSH and CAT, moreover, protecting heart and liver tissues.

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Our results are in agreement with the previous experimental study by (Aklil *et al*., 2017; El-Saleh *et al*., 2004; Haddadi *et al*., 2017). The latter reported that HHcy was induced by the high dose of L-methionine in mice and rats and confirmed the significant protective role of *Argania spinosa* extract, Thymoquinone and *Nigella sativa* oil and *lyophilized prune* extract against situational HHcy. In vitamin therapy, it could be also combined to an antioxidant treatment obtained from natural phytochemicals, a source for natural antioxidants to protect against the homocysteine mediated free oxygen radicals damages (Benmebarek *et al*., 2013). Recent studies have proven that antioxidants can lower Hcy levels very effectively (El-Saleh *et al*., 2004; Çelik *et al*., 2017). The study of (Labed *et al*., 2016) show that the ethyl acetate extract of *A. Armatus* contain a flavonoid and this extract exhibited the highest antioxidant activity in DPPH, ABTS and CUPRAC assays. Phenolic compounds as well as flavonoids are well-known as antioxidant, which are responsible for their health benefits, curing and preventing many diseases. (Tungmunnithum *et al*., 2018). The protective effect of *A. armatus* extract against situational HHcy is probably dueto its high content of flavonoid compound known as powerful antioxidant.

In addition, the administration of *A.armatus* extract with L-methionine caused a decrease in lipid parameterse, (Seo *et al*., 2010) proved that high intakes of antioxidants appeared to be protective factor against atherosclerosis, possiblyexerting a pro-oxidative effect on LDL when combined with high levels of Hcy and LDL. Recent evidence suggests that some polyphenols in their purified form, have beneficial effects on dyslipidemia in humans and animal models (Mulvihill and Huff, 2010).

Further, administration of *A. armanus* extrat in combination with methionine rich diet elevated plasma GSH and CAT levels, our results are in agreement with previous experimental research by (Aklil *et al*., 2017; El-Saleh *et al*., 2004; Çelik *et al*., 2017). The study by (Çelik*et al*., 2017; Meng *et al*., 2013) demonstrated that the quercetin treatment could have a preventive effect against oxidative stress produced by homocysteinemia in rats. One of the possible mechanisms of the protective effect of quercetin could be associated to its antioxidant properties, which rise antioxidant enzyme activities like SOD, CAT and GSH level, non-enzymatic antioxidant, in plasma. While the administration of *A.armatus* extract with L-methionine caused an improvement in histological changes, and corrected the structural abnormalities observed in the aorta and heart tissues, the work of (Aklil *et al*., 2017; Benmebarek *et al*., 2013) proved that, HHcy induced by the high methionine diet , could damage the aorta and heart tissue, and the treatment of these animals with *Argania spinosa* powdered seeds and *Stachys mialhesi* extract respectively corrected these alterations.

A.armatus extract has been confirmed to contain flavonoid wich indicated the highest antioxidant activity (Labed *et al*., 2016), The antioxidative characteristic of flavonoids are favored by a high degree of OH substitution. Furthermore, inhibition of enzymatic functions other than oxidases, like inhibition of lipoxygenase and thus protection of the formation of leukotrienes, may also participate in the cell and tissue preventive properties of flavonoids (De Groot and Rauen, 1998).

CONCLUSION

This current study proved that the extract of plant *Astragalus armatus* is effective in decreasing plasma homocysteine level, decline in lipid parameters, reduced oxidative stress by rising in antioxidant status, and protected the aorta and heart diseases in mice fed with L- methionine enriched diet.

This research is another step towards preventing the danger of HHcy, which has reduced the risk factor of cardiovascular diseases.

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Appendices

1- Treatment dose calculation

1-1 *armatus extract* given dose (100 mg/kg)

0.10 g ⟶ 1000g

X g ⟶ Mouse weight (g)

$$\text{A. armatus extract given dose } X = \frac{0.10\ \text{g} \times \text{Mouse weight (g)}}{1000\text{g}}$$

1-2 L- Methionine given dose (400 mg/kg)

0.40 g ⟶ 1000g

X g ⟶ Mouse weight g

$$\text{L- Methionine given dose } X = \frac{0.40\ \text{g} \times \text{Mouse weight (g)}}{1000\text{g}}$$

2- Preparation of PBS:

- Nacl : 8g
- Kcl : 0,2g
- NaH2PO4 : 1,15g
- KH2PO4 : 0,2g
- Mgcl2 6H 2O: 0,1g
- Cacl 2 2H 2O : 0,137g
- QS: 1 L of water

3- Hematoxylin eiosin staining:

1- Dip the lame in the alcohol solution for 5 minutes.

2- Rinse with tap water.

3- Immerse sections in Hematoxylin for 4 minute.

2. Rinse with tap water.

3. Exchange tap water until the water is clear.

4. Immerse sections in EOSIN stain for 10 minutes.

5. Rinse with tap water.

6. Exchange tap water until the water is clear.

7. Dehydrate in alcohol solutions for 1 min.

8. Clear with xylene.

العنوان

النشاط البيولوجي لنبتة *Astragalus armatus* على أمراض القلب والأوعية الدموية الناجمة عن فرط الهوموسستيين عند الفئران وعلى الخلايا السرطانية.

يرتبط فرط الهوموسيستين في الدم، الذي يتميز بزيادة مستوى الهوموسيستين في البلازما، بأمراض القلب والأوعية الدموية، تصلب الشرايين،الإصابات الكبدية، والتمثيل الغذائي غير الطبيعي للدهون، كما يعتبر أيضًا عامل خطر للإصابة بالسرطان. أكدت العديد من الدراسات انخفاض مستوى الهوموسيستين في البلازما عن طريق العديد من المركبات المضادة للأكسدة الفينولية والمستخلصات النباتية. أظهرت الدراسات أن بعض مركبات الفلافونويد يمكن أن تساهم في الوقاية من السرطان وهي فعالة بشكل خاص في منع تكاثر الخلايا السرطانية.

لقد أجرينا هذا البحث لتقييم التأثير الوقائي لمستخلص الإثيل أسيتات (AcOEt) لنبتة *A. armatus* على أمراض القلب والأوعية الدموية بعد إحداث حالة فرط الهوموسيستيين، و أيضا تقييم النشاط المضاد للأكسدة والنشاط المضاد للتكاثر للمستخلص البوتانولي (n-BuOH) لنبتة *A. armatus* في العضوية.

أدى استهلاك نظام غذائي عالي من الميثيونين ﴿400 ملغ/كلغ﴾ خلال 21 يومًا إلى إلى زيادة معتبرة في الهوموسيستيين في البلازما. علاوة على ذلك، أدى إلى زيادة في تركيزات الدهون، وانخفاض في HDL-c، الجلوتاثيون (GSH) و الكاتلاز (CAT). بالإضافة إلى ظهور تغيرات و تشوهات نسيجية في الشريان الأورطي، القلب و الكبد. في حين أن إعطاء مستخلص نبتة *A. armatus* مع الميثيونين أدى إلى انخفاض في تركيز كل من الهوموسيستيين، الدهون، وزيادة في نشاط الجلوتاثيون و الكاتالاز، مع تحسين التغيرات و التشوهات النسيجية .كما بينت نتائجنا أن تقييم النشاط المضاد للأكسدة خارج العضوية لمستخلص الكسر البوتانولي لنبتة *A. armatus* بأربع طرق، أعطى نشاطا منخفضا. بينما بينت نتائج التقييم في المختبر للنشاط المضاد للتكاثر لمستخلص الكسر البوتانولي لنبتة *A. armatus* ضد خلايا HeLa سرطان عنق الرحم البشري وخلايا C6 ورم دماغ الجرذان، أن استعمال تراكيز مختلفة من هذا المسخلص يؤدي إلى تثبيط النمو والنشاط التكاثري للخلايا السرطانية مثل خلايا HeLa وخلايا C6، بالإضافة إلى ذلك، تم تقييم مستخلص الكسر البوتانولي لنبتة *A. armatus* أنه يحتوي على أنشطة عالية مضادة للتكاثر ضد خلايا C6 مقارنة بخلايا HeLa عند التركيز100 ميكروغرام/مل.

أظهرت النتائج المتحصل عليها في هذه الدراسة أن نبات *A. armatus* يمكن اعتباره مصدرًا طبيعيًا للوقاية من أمراض القلب والأوعية الدموية والسرطان.

الكلمات المفتاحية: فرط الهوموسيستين في الدم، أمراض القلب والأوعية الدموية، *Astragalus armatus*، مضادات الأكسدة الإنزيمية، النشاط المضاد للأكسدة، النشاط المضاد للتكاثر، خلايا HeLa وخلايا C6 .

Activité biologique d'Astragalus armatus sur les maladies cardiovasculaires induites par l'hyperhomocystéinémie chez la souris et sur les lignées cellulaires cancéreuses.

Le résumé:

L'hyperhomocystéinémie (HHcy), caractérisée par une augmentation du taux plasmatique d'homocystéine (Hcy), liée aux maladies cardiovasculaires, à l'athérosclérose, aux lésions hépatiques et au métabolisme lipidique anormal, est également un facteur de risque de cancer. De nombreuses études ont confirmé la réduction du taux plasmatique d'Hcy par plusieurs composés antioxydants phénoliques et extraits de plantes. Des études ont montré que certains flavonoïdes peuvent contribuer à la prévention du cancer et sont particulièrement efficaces pour inhiber la prolifération des cellules tumorales.

Nous avons mené cette recherche pour confirmer et évaluer l'effet protecteur de l'extrait d'acétate d'éthyle de la plante *A. armatus* sur les maladies cardiovasculaires induites par l' HHcy chez la souris, causée par la forte dose de L-méthionine. D'autre part, évaluation de l'activité antioxydante et de l'activité antiproliférative de l'extrait n-butanol de la plante *A. armatus* in vitro.

La consommation d'un régime riche en L-méthionine (400mg/Kg) pendant 21 jours conduit à une augmentation de l'Hcy plasmatique, de même des concentrations des paramètres lipidiques et une diminution des activités du HDL-c, du glutathion réduit (GSH) et de la catalase (CAT). Celles-ci étaient associées à l'apparition d'altérations pathologiques de l'aorte, du cœur et des organes hépatiques. Tandis que l'administration de l'extrait d'acétate d'éthyle de la plante *A. armatus* (100mg/Kg) avec L-méthionine (400mg/Kg) a provoqué: une diminution de la concentration en Hcy et des paramètres lipidiques, une augmentation des activités GSH et CAT, et une amélioration des modifications histologiques. Néanmoins, les résultats de l'évaluation de l'activité antioxydante in vitro de l'extrait n-butanol de la plante *A. armatus* par quatre méthodes ont montré une faible activité.Alors que les activités antiprolifératives de l'extrait n-butanol de la plante *A. armatus* ont montré une augmentation des activités en fonction de l'augmentation de la dose contre les cellules HeLa et C6. De plus, il a été déterminé que l'extrait n-butanol de la plante *A. armatus* avait des activités antiprolifératives plus élevées contre les cellules C6 que contre les cellules HeLa à toutes les concentrations.

Les résultats obtenus dans cette étude montrent que la plante *A. armatus* peut être considérée comme une source naturelle pour prévenir les maladies cardiovasculaires et cancéreuses.

Mots clés: Hyperhomocystéinémie, Maladies cardiovasculaires, *Astragalus armatus*, Enzymes antioxydantes, Activité antioxydante, Cellules HeLa et C6.

Abstract:

Hyperhomocysteinemia (HHcy), characterized by an increase in the plasma level of homocysteine (Hcy), is related with cardiovascular disease, atherosclerosis, hepatic lesions and abnormal lipid metabolism, is also a risk factor for cancer. Many studies have confirmed the discount of plasma Hcy level by several phenolic antioxidant compounds and plant extracts. Studies have shown that some flavonoids can contribute to cancer prevention and are particularly effective at inhibiting tumor cell proliferation.

We have conducted this research to confirm and evaluate the protective effect of ethyl acetate extract of the *A. armatus* plant on on cardiovascular diseases induced by hyperhomocysteinemia in mice, caused by the high dose of L-methionine. On the other hand, evaluation of antioxidant activity and antiproliferative activity of n- butanol extract of *A. armatus* plant in vitro.

Consumption of high L-methionine (400mg/Kg) diet during 21days, led to an increase in plasma Hcy, lipid parameters concentrations, and a decrease in HDL-c, glutathione reduced (GSH) and catalase (CAT) activities. These were associated with the appearance of pathological alterations in the aorta, the heart and the liver organs. While the administration of ethyl acetate extract of the *A. armatus* plant (100mg/Kg) with L-methionine (400mg/Kg) caused: a decrease in Hcy concentration and lipid parameters, an increase in GSH and CAT activities, and an improvement in histological changes. However the results of the evaluation of antioxidant activity in vitro of n- butanol extract of the *A. armatus* plant by four methods showed a low activity.

While the antiproliferative activities of n- butanol extract of the *A. armatus* plant, was shown to increase of the activities depending to dose increasing against HeLa and C6 cells. In addition to, n-butanol extract of the *A. armatus* plant was determined to have the higher antiproliferative activities against C6 cells than against HeLa cells at the concentration 100 µg/mL.

The results obtained in this study show that the plant *A. armatus* can be considered as natural source in the prevention against cardiovascular and cancer diseases.

Keywords: Hyperhomocysteinemia, Cardiovascular diseases, *Astragalus armatus*, Antioxidant enzymes, Antioxidant activity, HeLa and C6 cells.

Family Name: BAGHRICHE

First Name: Ibtissam

Academic year: 2023-2024

Title: Biological activity of *Astragalus armatus* on cardiovascular diseases induced by Hyperhomocysteinemia in mice and on cancer cell lines.

Thesis submitted for the degree of DOCTORAT IN SCIENCES

Hyperhomocysteinemia (HHcy), characterized by an increase in the plasma level of homocysteine (Hcy), isrelated with cardiovascular disease, atherosclerosis, hepatic lesions and abnormal lipid metabolism, is also arisk factor for cancer. Many studies have confirmed the discount of plasma Hcy level by several phenolic antioxidant compounds and plant extracts. Studies have shown that some flavonoids can contribute to cancer prevention and are particularly effective in inhibiting tumor cell proliferation.

We have conducted this research to confirm and evaluate the protective effect of ethyl acetate extract of the *A. armatus* plant on on cardiovascular diseases induced by hyperhomocysteinemia in mice, caused by the high dose of L-methionine. On the other hand, the antioxidant and antiproliferative activities of n-butanol extract of *A. armatus* plant was carried *in vitro*.

Consumption of high L-methionine (400mg/Kg) diet during 21days, led to an increase in plasma Hcy, lipid parameters concentrations, and a decrease in HDL-c, glutathione reduced (GSH) and catalase (CAT) concentrations. These were associated with the appearance of pathological alterations in the aorta, the heart and the liver organs. While the administration of ethyl acetate extract of the *A. armatus* plant (100mg/Kg) with L-methionine (400mg/Kg) caused a decrease in Hcy concentration and lipid parameters, an increase in GSH and CAT activities, and an improvement in histological changes. However we have obtained a low antioxidant activity with n- butanol extract of the *A. armatus in vitro*

While the antiproliferative activities of n- butanol extract of the *A. armatus* plant, was shown to increase the activities depending on the dose manner against HeLa and C6 cells. In addition, n- butanol extract of the *A. armatus* plant was determined to have the higher antiproliferative activities against C6 cells than HeLa cells at at the concentration 100 µg/mL.

The results obtained in this study show that the plant *A. armatus* can be considered as natural source in the prevention against cardiovascular and cancer diseases.

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