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Biological activities of *Vitis vinifera* leaves in cardiovascular diseases induced by hyperhomocysteinemia and on tumoral process

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Dedication

Is my genuine gratefulness and warmest regard that I dedicate this thesis to my family especially to my parents.

Selma

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List of publication

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2. Selma Ferhi, Sara Santaniello, Sakina Zerizer, Sara Cruciani, Angela Fadda, Daniele Sanna, Antonio Dore, Margherita Maioli and Guy D'hallewin. Total Phenols from Grape Leaves Counteract Cell Proliferation and Modulate Apoptosis-Related Gene Expression in MCF-7 and HepG2 Human Cancer Cell Lines. Molecules 2019, 24, 612.

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List of abbreviation

%	Percent	**€**	Euro
		ATP	Adenosine Triphosphate
°C	Degré Celsius	**µg**	Microgram
4-HNE	4-HydroxyNonEnal	**5,10-MTHFR**	5,10-Methylene Tetrahydrofolate Reductase
5-MTHF	5-MethylTetraHydroFolate	**ADMA**	Asymmetric dimethylarginine
Ang	Angiotensin	**ANOVA**	Analyse of Variance
AP-1	Activator protein-1	**ASE**	Accelerated Solvent Extraction
BAX	Bcl-2-associated x	**BCL-2**	B cell lymphoma 2
CO_2	Carbone Dioxide	**COMT**	Catechol-O-MethylTransferase
CßS	Cystathionine Synthase	**CGL**	Cysteine Lyase
CVD	Diseases of the circulatory system	**DMSO**	DiMethylSufOxide
DAD	Diode Array Detector	**DPBS**	Dulbecco's Phosphate Buffered Saline
DNA	Deoxyribonucleic acid	**DTNB**	5,5′-DiThiobis-(2 Nitro-Benzoic acid)
DPPH•	2,2-Diphenyl-1-icrylhydrazyl	**EACE**	Ethanolic ASE Crude Extract
DW	Dry weight	**ER**	Endoplasmic Reticulum
eNOS	Nitric Oxide Synthase	**ESI**	ElectroSpray Interface
ERP-spin	Electron Paramagnetic Resonance (EPR)	**FBS**	Fetal Bovine Serum
EU	Europe	**GAE**	Gallic Acid Equivalent
G	Gramme	**GPX**	Peroxidase Glutathione
GLAV	Grape leaves Algerian variety	**GSH**	Gluthatione Reduced
GRP78	Glucose Regulated Protein-78	**H**	Hour
GSSG	Oxidized Glutathione	**HCl**	HydroChloric acid
H_2O_2	Hydrogen peroxide	**Hcy**	L-Homocysteine
Hcy	Homocysteine	**HGAPDH**	Glyceraldehyde-3-Phosphate Dehydrogenase
HepG2	Liver hepatocellular cells G2	**HPLC-DAD/ESI-MS**	High-Performance Liquid Chromatography-Diode Array Detection-Electrospray Ionization/mass spectrometry
HO•	Hydroxyl Radical	**HUVEC**	Human umbilical vein endothelial cell
hs-CRP	High Sensibility C-Reactive Protein Test	**IC50**	Inhibitrice Concentration 50
HVA	HomoVanilic Acid	**LSGS**	Low Serum Growth Supplement
L	Litre	**MAO**	Monoamine oxidase
m/z	Mass/charge	**MCF-7**	Human breast adenocarci-

			noma cell line
MAT	Methionine Adenosyl Transferase	**MS**	Methionine Synthase
mRNA	RiboNucleic Acid messager	**NaCl**	Sodium Chloride
MTT	3-(4,5-diMethylThiazol-2-yl)-2,5-diphenylteTrazolium bromide] tetrazolium	**NADPH**	Nicotinamide Adenine Dinucleotide Phosphate
NADH	Nicotinamide Adenine Dinucleotide	**NMN**	NorMetaNephrine
Nm	Nano metre	**NO**	Nitric Oxide
NO	Nitric Oxide	**O_2 •**	Superoxide Radical
NO_2	Azote dioxide	**O_3**	Ozone
O_2	Oxygen	**PBS**	Phosphate Buffer Saline
ONOO-	Peroxynitrite	**PGP-49**	Glucose Regulated Protein-49
PCR	Polymerase Chain Reaction	**Psi**	Pound-force/square inch
PO_2	Intracellular oxygen tension	**RNA**	Ribonucleic Acide
PTFE	PolyTetraFluoroEthylene	**RPLC**	Reverse Phase Liquid Chromatography
ROS	Reactive Oxygen Species	**SAH**	S-Adenosine-L-Homocysteine
S	Second	**SAM**	S-Adenosyl-L-Methionine
SAH	S-adenosylhomocysteine	**SOD**	Superoxide Dismutase
SD	Standard Deviation	**TNF*α***	Tumor Necrosis Factor
SPSS	Statistical Package for the Social Sciences	**Tr**	Retention time
tHyc	Plasma total Homocysteine	**US**	United state
TP	Total Phenolic	**V**	Volume
tR	Retention time	**VCAM-1**	Vascular cell adhesionMolecule
UV	Ultraviolet	**w/v**	weight/volume
v/v	Volume/volume	**Ml**	Microlitre
VMA	Vanillyl-mandelic acid	**Min**	Minute
WACE	Water ASE crude extract,		

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Introduction

Introduction

Utilization of plants for medicinal purposes can be traced back to the beginning of human history. Use of plants as healing agents is already depicted in the paintings discovered in the Lascaux caves in France, which have been dated between 13,000 and 25,000 B.C. (Berger, 2006). Herbal medicine even appears to have its roots within the animal kingdom as there is now convincing evidence for self-medication in non-human primates. Thus, it has been observed that chimpanzees, bonobos and gorillas swallow leaves and chew bitter piths to treat parasitoses and related illnesses. Most interestingly, the plants used by these apes are identical to those applied by local human populations for their own medication (Huffman, 2006).

In developed countries, Diseases of the heart and circulatory system (CVD) are the leading cause of mortality in the world and Europe as a whole, responsible for over 3.9 million deaths a year, or 45% of all deaths. CVD has major economic costs as well as human costs for Europe. Overall CVD is estimated to cost the EU economy €210 billion a year. Of the total cost of CVD in the EU, 53% (€111 billion) is due to direct health care costs, 26% (€54 billion) to productivity losses and 21% (€45 billion) to the informal care of people with CVD (You et al., 2012 and Wilkins et al., 2017).

Hyperhomocysteinemia is defined as an abnormally high plasma homocysteine (Hcy) concentration after an oral Methionine load (Van Den Berg et al., 1995). It is a factor of risk for premature cardiovascular disease (Willems et al., 2002). Hence, it is one of the major pathogenic factors of atherosclerosis (Boldyrev et al., 2009). Besides its detection in all inflammatory diseases, hyperhomocysteinemia has been reported in other sicknesses like: type 2 diabetes, chronic kidney disease and cancer (Falvoa, 2007 and Wu, 2008), and Alzheimer (Morris et al., 2001). It should be mentioned that hyperhomocysteinemia is not produced only by inflammation, but also by oxidative stress generated by high plasma homocysteine, which can cause a hyperhomocysteinemia induced inflammation (Jacobsen, 2000).

In addition, the relationship between hyperhomocysteinemia and cardiovascular disease is highlighted by the deficiency of the cystathionine beta-synthase (CβS) enzyme, which is deficient during homocysteinuria (Flemming et al., 2010). In most cases, hyperhomocysteinemia is a result of deficiency of the vitamins B6, B12, folate, or a combination of them (Chiang et al., 2005). These vitamins are essential co-factors of the key enzymes of the Homoysteine's metabolism. Moreover, some drugs such as fibrates, antiepileptic, methotrexate, theophylline, metformin, and other substances like nicotinic

acid can also cause hyperhomocysteinemia (Stalder et al., 2010). Homocysteine acts directly on endothelial and damaged vessel wall through generating an oxidative stress, and stimulating a pro-coagulant and pro-inflammatory state of blood components (Bernardo et al., 2004) is the most accepted hypothesis about Hcy's action in cardiovascular disease. Several studies have demonstrated that correcting the plasma deficiency of folic acid and vitamin B12 decreases or makes hyperhomocysteinemia disappear (Rigaud, 1999).

Moreover, Cancer is the major cause of morbidity and mortality in modern society. The number of deaths by cancer in 2008 was estimated to be 7.6 million, a number predicted to double by 2030 (Ferlay et al., 2010). Many treatments against cancer are possible, such as surgical removal, chemotherapy, radiation therapy and immunotherapy.

Further, Apoptosis, or programmed cell death, is a normal and fundamental event that occurs in a highly regulated and precise manner. This process plays a key role in normal tissue development and maturation, maintaining the homeostasis in the body by controlling the immune system. Apoptosis is the most potent defence against cancer since it is the mechanism used by metazoans to eliminate deleterious cells. Furthermore, a large number of chemo preventive agents exert their effectiveness by inducing apoptosis in transformed cells, as shown both *in vitro* and *in vivo* (Sun et al., 2004 and Fresco et al., 2010). Since apoptosis provides a physiologic mechanism to eliminate abnormal cells, dietary factors affecting apoptosis can elicit an important effect on carcinogenesis. For these reasons, activation of apoptosis by dietary factors in pre-cancerous cells may represent a preventive mechanism (chemoprevention) (Martin, 2006 and Fresco et al., 2010).

Now, Drugs derived from natural resources represent a significant segment of the pharmaceutical market as compared to randomly synthesized compounds. Phytotherapy, whose therapeutic efficacy is based on the combined action of a mixture of constituents, offers new treatment opportunities. Because of their biological defense function, plant secondary metabolites act by targeting and disrupting the cell membrane, by binding and inhibiting specific proteins or they adhere to or intercalate into RNA or DNA. Phytotherapeutics may exhibit pharmacological effects by the synergistic or antagonistic interaction of many phytochemicals. Mechanistic reasons for interactions are bioavailability, interference with cellular transport processes, activation of pro-drugs or deactivation of active compounds to inactive metabolites, action of synergistic partners at

different points of the same signaling cascade (multi-target effects) or inhibition of binding to target proteins (Thomas and Egon, 2011).

The term "Nutraceuticals" was coined in 1989 by Stephen De Felice to define "food, or parts of a food, that provide medical or health benefits, including the prevention and treatment of disease" (Kalra, 2003, Basoli et al., 2017 and Santaniello et al., 2018). *Vitis vinifera* leaves have been traditionally used as food or as medications all over the world. The leaves are used to treat hypertension, diarrhea, hemorrhage and varicose veins, inflammatory disorders, and reduce blood glucose levels in diabetics (Dani et al., 2010). Moreover, the use of grape leaves provides a way of solving the disposal problems arising from the large amounts of industrial residues generated by the wine and juice industries (Monagas et al., 2006 [a,b]).

Correspondingly, extraction of Nutraceuticals is the most important step to recover and isolate bioactive molecules from plant materials. Various extraction techniques have been developed to obtain nutraceuticals from plants in order to shorten extraction time, reduce solvent consumption, increase extraction yield, improve the quality of extracts and increase pollution prevention (Wang and Weller, 2006). Among those, accelerated solvent extraction (ASE) is a solid-liquid extraction process performed at elevated temperature and under pressure to maintain the solvent in its liquid state. The solvent remains below its critical condition during ASE. The increased temperature accelerates the extraction kinetics and the elevated pressure keeps the solvent in the liquid state, thus achieving a safe and rapid extraction. The only disadvantage of ASE is the high cost of the needed equipment (Romanik et al., 2007).

Our objectives in this study were to:

1. Purification of phenolic compounds of grape leaves Algerian variety (Anthocyanins and non anthocyanins) by using HPLC-DAD/ESI-MS;

2. Measuring the total Hyc, the plasma hs-CRP and the concentration of the GSH to estimate the antioxidant and anti-inflammatory effect of the grape leaves Algerian variety on the inflammation induced by Hyperhomocysteinemia;

3. The aorta histology had been examined in order to confirm the angiotoxic action of homocysteine and the effect of grape leave Algerian variety on the aorta.

4. Optimised the quantity of polyphenol extracted from grape leaves by using Accelerator Solvant Extract;

5. Measured the total phenolic content by using the modified Folin–Ciocalteau;

6. Evaluated the antioxidant activity by using two different methods2,2-diphenyl-1-picrylhydrazyl (DPPH•) free radical-scavenging and Spin trapping assay of the •OH radical;

7. Estimated the anti-proliferative by using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] tetrazolium reduction assay of grape leaves extract on breast cancer and hepatocarcinoma by Modulate Apoptosis-Related Gene Expression of the report of Bax/Bcl-2.

Chapter I Literature Review

I.1 Oxidative stress

I.1.1 Definition

Oxidive stress is defined as an imbalance between productions of free radicals and reactive metabolites, so-called oxidants or reactive oxygen species (ROS), and their elimination by protective mechanisms, referred to as antioxidants. This imbalance leads to damage of important biomolecules and cells, with potential impact on the whole organism. ROS are products of a normal cellular metabolism and play vital roles in stimulation of signalling pathways in plant and animal cells in response to changes of intra- and extracellular environmental conditions (Reuter et al., 2010).

I.1.2 Reactive oxygen species (ROS)

The term "ROS" includes all unstable metabolites of molecular oxygen (O_2) that have higher reactivity than O_2 like superoxide radical (O_2 ••) and hydroxyl radical (HO•) and non-radical molecules like hydrogen peroxide (H_2O_2). These ROS are generated by product of normal aerobic metabolism, but their level increases under stress which proves to be a basic health hazard. Mitochondrion is the major cell organelle responsible for ROS production. It generates ATP through a series of oxidative phosphorylation processes. During this process, one or two-electron reductions instead of four electron reductions of O_2 can occur, leading to the formation of O_2 • or H_2O_2 and these species can be converted to other ROS (Figure 1). Other sources of ROS may be reactions involving peroxisomal oxidases, cytochrome P-450 enzymes, NAD (P)H oxidases, or xanthine oxidase (Favier, 2003) .

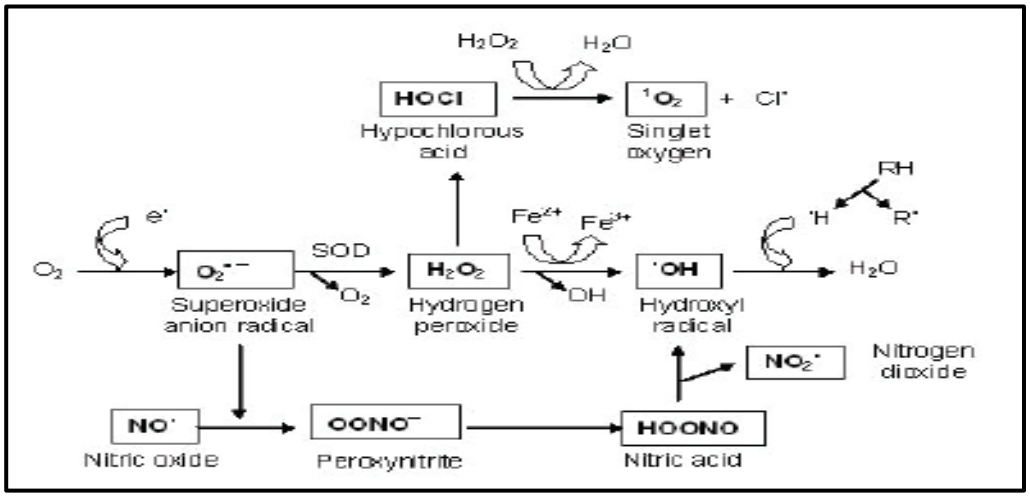

Figure 1: Mutual association between free radicals and their reactive metabolites (Ďurackova, 2008).

I.1.3 The antioxidants

The body has a complex set of antioxidant defenses to protect against the deleterious effects of ROS (Haleng et al., 2007). Antioxidants now appear as the keys to longevity and our allies to fight against modern diseases. These are protective elements that act as free radical scavengers (Bartosz, 2003). Antioxidants are defined as "any endogenous or exogenous substance which in low concentration relative to the oxidizable substrate prevents or slows the oxidation of that substrate" (Pastre and Priymenko, 2007), by the release of one or more electrons. Some antioxidants are manufactured by the body such as enzymes (Figure 2) (superoxide dismutase SOD, glutathione peroxidase and reductase, catalase), proteins (ferritin, transferrin, ceruloplasmin and albumin), others come from food rich in vitamins C, E, carotenoids, ubiquinone (coenzyme Q10), flavonoids, polyphenols, glutathione, histidine dipeptide or lipoic acid (Pokorny et al., 2001). Added to this, some as trace elements such as selenium, copper and zinc which are cofactors of antioxidant enzymes and uric acid and bilirubin (Haleng et al., 2007). Both in natural form or in the form of additives used in the food industry (Tanguy, 2009), which has a greater heterogeneity such as vitamins, minerals and secondary metabolites (phenolic compounds). Others are both synthesized in small quantities by the body and brought by food. This is the case, for example, with cysteine and Coenzyme Q10 (Pokorny et al., 2001). There is currently arenewed interest in phytochemicals as sources of natural antioxidants. The goal is to use them in foods and pharmaceutical preparations to replace synthetic antioxidants, which cause potential health risks due to their carcinogenic or mutagenic effects (Le Cren, 2004).

In addition, they are less well absorbed by our bodies than those from natural sources. This is the case, for example, with vitamin E. Studies have shown that synthetic vitamin E blocks the activity of this natural vitamin at the cellular level (Pelli and Lyly, 2003).

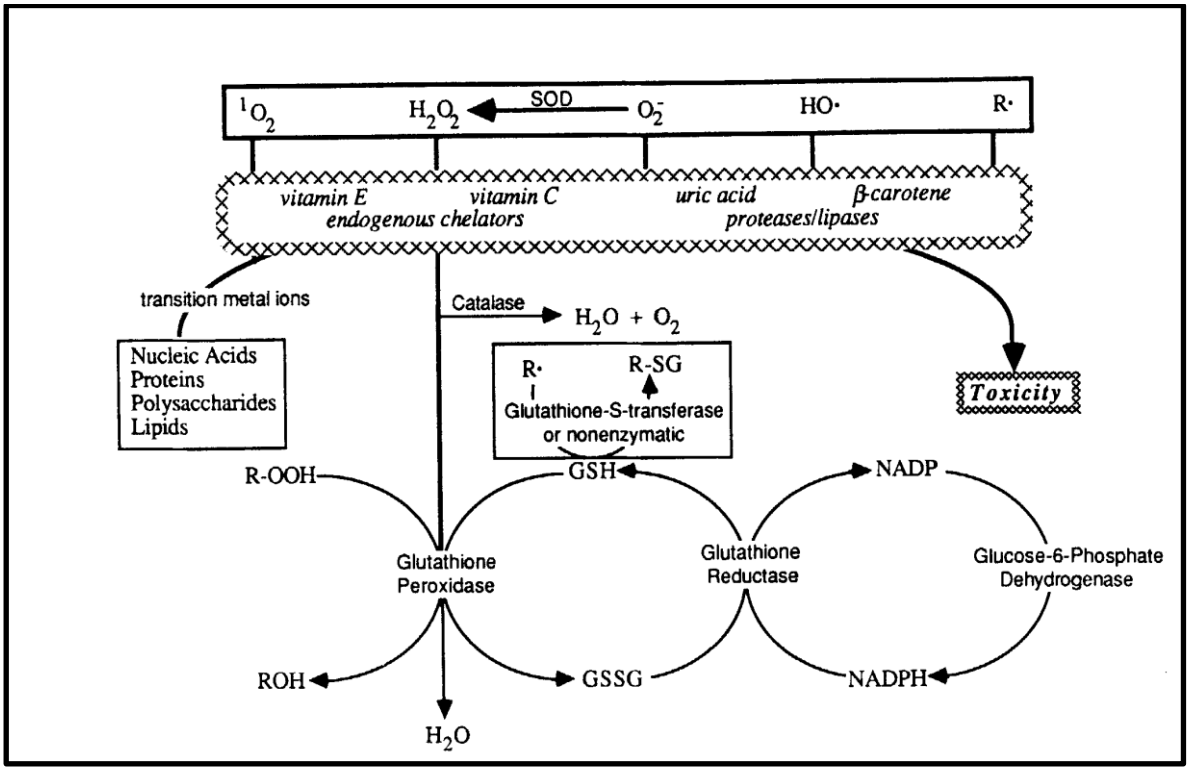

Figure 2: Biological antioxidant defense systems. All aerobic cells contain a spectrum of chemical and enzymatic antioxidants that works in concert to minimize undesirable oxidative reactions within cells. SOD: superoxide dismutase; GSH: reduced glutathione; GSSG: glutathione disulphide (Kehrer, 1993).

I.1.4 Oxidative stress and human diseases

Increased oxidative damage has been reported for almost all known diseases. Therefore, a clear correlation between disease and oxidative stress is far from being proven for most pathological conditions. Disorders in which oxidative stress was demonstrated to be a key determinant in the pathogenic process are essentially limited to very few examples, such as chronic deprivation of selenium and reduced intake of vitamin E due to diseases affecting intestinal fat absorption (e.g. abetalipoproteinemia) (Granot and Kohen, 2004 and Brenneisen et al., 2005). In general, pathologies related to an altered homeostasis of metals, such as Wilson's disease (due to copper overload) and thalassemia (related to iron overload), are strictly linked to oxidative stress since these metal ions are known to induce generation of ROS (Ferenci, 2004 and Rachmilewitz et al., 2005). Similarly, exposure to exogenous factors, such as ionizing radiation, elevated PO_2, ultraviolet light, and high doses of a mixture of air pollutants, such as NO_2 and O_3, favours the generation of ROS, which, in turn, are thought to be responsible for the onset of inflammatory processes, mainly in the respiratory airways (Churg, 2003). In other diseases, the link between

oxidative stress and the pathogenic process may be inferred and an increased production of RONS can reasonably be hypothesized (Figure 3). However, a convincing link between the occurrence of increased oxidative stress and the development and/or progression of disease has not yet been clearly demonstrated (Giustarini et al., 2009).

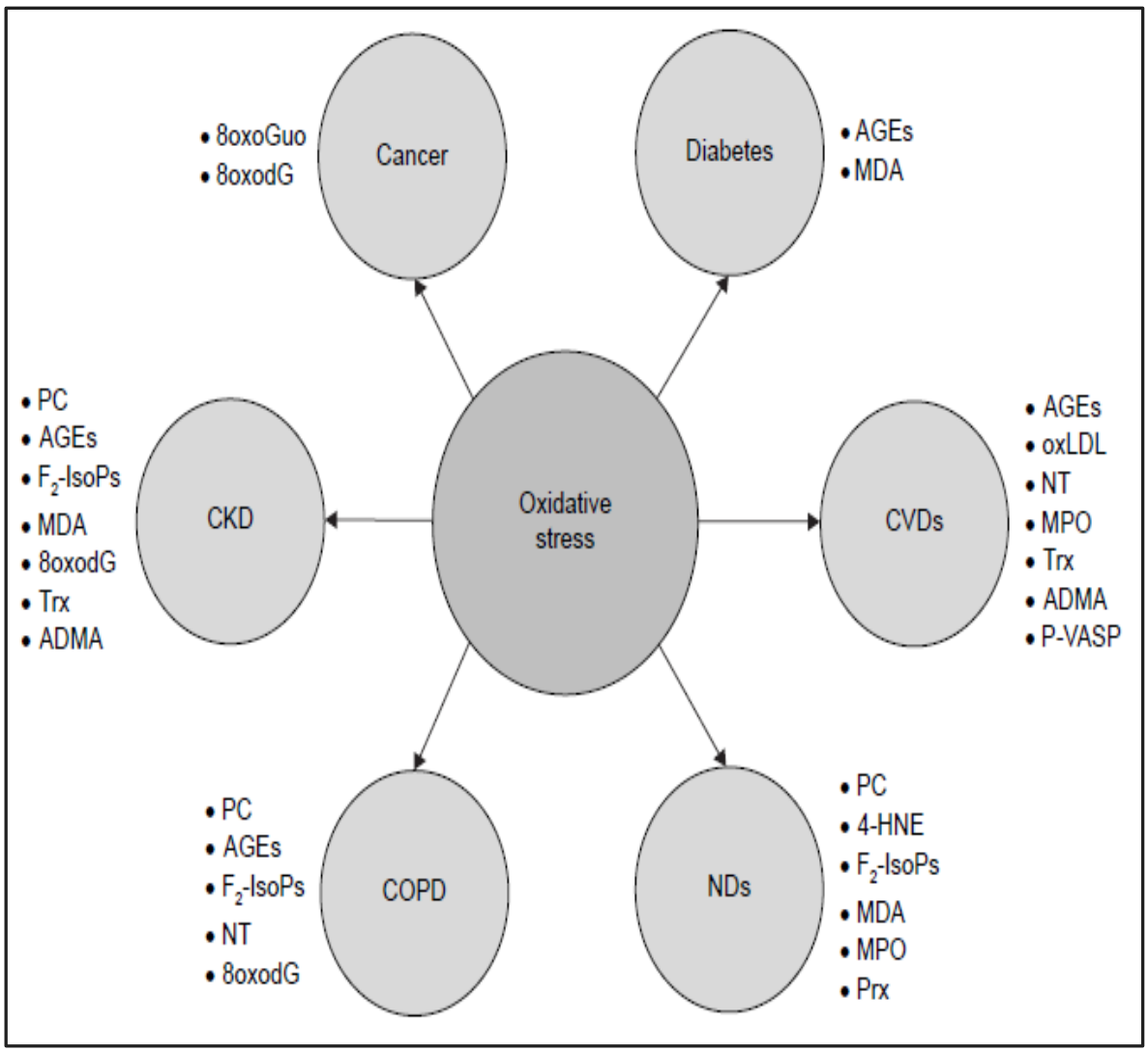

Figure 3: Oxidative stress, age-related diseases, and relative biomarkers (Liguori et al., 2018).

4-HNE, trans-4-hydroxy-2-nonenal; 8oxodG, 7,8-dihydro-8-oxo-2′-deoxyguanosine; 8oxoGuo, 7,8-dihydro-8-oxoguanosine; ADMA, asymmetric dimethyl l-arginine; AGEs, advanced glycation end products; CKD, chronic kidney disease; CVDs, cardiovascular diseases; F2-IsoPs, F2-isoprostanes; MDA, malondialdehyde; MPO, myeloperoxidase; NDs, neurodegenerative diseases; NT, nitrotyrosine; oxLDL, oxidized low-density. lipoprotein; PC, protein carbonyl; Prx, peroxiredoxins; P-VASP, phosphorylated vasodilator-stimulated phosphoprotein; Trx, thioredoxin.

I.2 Inflammation

I.2.1 Definition:

The word inflammation comes from the Latin "inflammo", meaning "I set alight, I ignite»; it's defined as a series of protective and regenerative responses of the body (Teruki et *al*., 2014). Which participates importantly in host defences against infectious agents and injury, but it also contributes to the pathophysiology of many chronic diseases. Interactions of cells in the innate immune system, adaptive immune system, and inflammatory mediators orchestrate aspects of the acute and chronic inflammation that underlie diseases of many organs. A coordinated series of common effector mechanisms of inflammation contribute to tissue injury, oxidative stress, remodelling of the extracellular matrix, angiogenesis, and fibrosis in diverse target tissues (Peter, 2007).

I.2.2 Network inflammation and oxidative stress

Oxidative stress can influence many biological processes such as apoptosis, viral proliferation, and inflammatory reactions. In these processes, gene transcription factors such as nuclear factor NF-κB and activator protein-1 (AP-1) act as oxidative stress sensors through their own oxidation and reduction cycling (Yoshikawa and Naito, 2002).

In the case of inflammation, TNF-α stimulate the activation of caspase- 3 and the apoptotic pathway, the liaison TNF-α with TNFR stimulate and activate NF-κB by the inhibition of a protein, IκBα, that normally binds to NF-κB and inhibits its translocation, is phosphorylated by IKK and subsequently degraded, releasing NF-κB. The transcription factor (NF-κB) is a heterodimeric transcription factor that translocate to the nucleus and mediates the transcription of a vast array of proteins involved in inflammatory and pro-apoptotic response (Taib et al., 2016). Inducers of NF-kB activation, such as tumor necrosis factor-alpha (TNF-a), phorbol 12-myristate 13-acetate, or UV radiation cause oxidative stress, suggesting that the induction of radical oxygen species (ROS) is a common signal to a wide variety of NF-kB–inducing conditions. Angiotensin (Ang) II, the main effector of the renin-angiotensin system, plays an essential role in the regulation of blood pressure but is also involved in remodeling of the arterial wall. It has recently been shown that Angiotensin II receptor type 1 (Ang II AT1) receptors decrease the formation of the atheromatous plaque in several animal models of atherosclerosis and that Ang II increases leukocyte adhesion to the endothelium *in vitro*. Given that VCAM-1 expression

in endothelial cells is inhibited by AT1 receptor antagonists *in vivo*, we hypothesized that Ang II could directly modulate VCAM-1 expression in these cells. Indeed, endothelial cells express AT1 receptors, which activate different intracellular path- ways16 and modulate several cell functions. Moreover, Ang II stimulates the generation of ROS in smooth muscle cells via the activation of NADH/NADPH oxidases and could be also implicated in ROS production in endothelial cells (Pueyo et al., 2000).

I.2.3 Homocycteine

I.2.3.1 Definition:

Homocysteine is a thiol amino acid, but only a small fraction (< 2%) of plasma tHcy circulates in the thiol form. The remainder is a mixture of disulfide derivatives, including homocystine, homocysteine-cysteine mixed disulfide, and protein-bound disulfides (Ganguly and Alam, 2015).

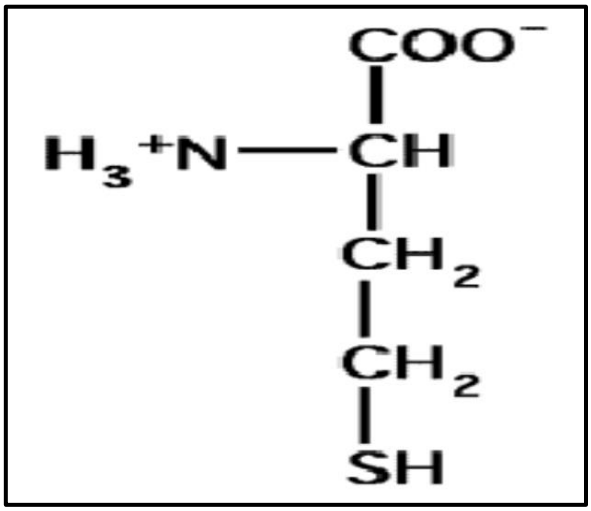

Figure 4: Structure of hymocycteine (Ganguly and Alam, 2015)

I.2.3.2 Metabolism of Homocysteine

L-Homocysteine (Hcy) is a sulfur-containing amino acid formed intracellularly from the diet-fed methionine. Hcy is not genetically coded. It is synthesized by all the cells of the body. The catabolism of Hcy occurs mainly in the liver and kidneys by two routes: the remethylation pathway and the transulphurization pathway (Biroulet, 2008).

I.2.3.2.1 The pathways of remethylation

The remethylation pathways provide for the methylation of Hcy to methionine by two separate enzymatic reactions. The main reaction involves two enzymes: the 5-MethylTetraHydroFolate (5-MTHF), a methyl-forming donor, is under the control of an enzyme, 5,10-MTHFR (5,10-Methylene Tetrahydrofolate Reductase), and Methionine Synthase (MS) whose cofactor is vitamin B12 (Figure 5).

This transfer of the methyl group, which allows the synthesis of methionine, is possible only in the presence of methylcobalamin, hence the synergy of action between vitamin B9 and vitamin B12. Second reaction takes place largely at the level of the liver. It is of low activity, and involves a liver enzyme, beta-homocysteine methyl-transferase. The Betaine is the donor methyl group molecule. The relative importance of these two remethylation pathways varies with tissue and protein status (Biroulet, 2008).

I.2.3.2.2 The way of transsulfuration

The majority of Hcy is not remethylated but catabolised into cysteine by the way of the transsulfuration. This pathway allows methionine, to bring a sulfur atom for the formation of cysteine. Activation of methionine to S-Adenosyl-L-Methionine (SAM) is under the influence of Methionine Adenosyl Transferase (MAT).

The SAM, the main donor of the methyl group of the organism, then yields this group to give rise to S-Adenosine-L-Homocysteine (SAH). This molecule is hydrolyzed to adenosine and homocysteine by S-Adenosyl-L- Homocysteine Hydrolase (Figure 5).

Under the influence of Cystathionine Synthase (CßS), Hcy then condenses with serine to form Cystahionine, itself cleaved and delaminated into cysteine and cetobutyrate (by Cysteine Lyase (CGL). Reactions require the presence of an enzymatic cofactor, pyridoxal phosphate or vitamin B6. Unlike other metabolic pathways, the latter is irreversible, and with the result that cysteine cannot be a precursor for methionine synthesis. This fact is especially important in the context of dietary recommendations (Chen, 2009) (Figure 5).

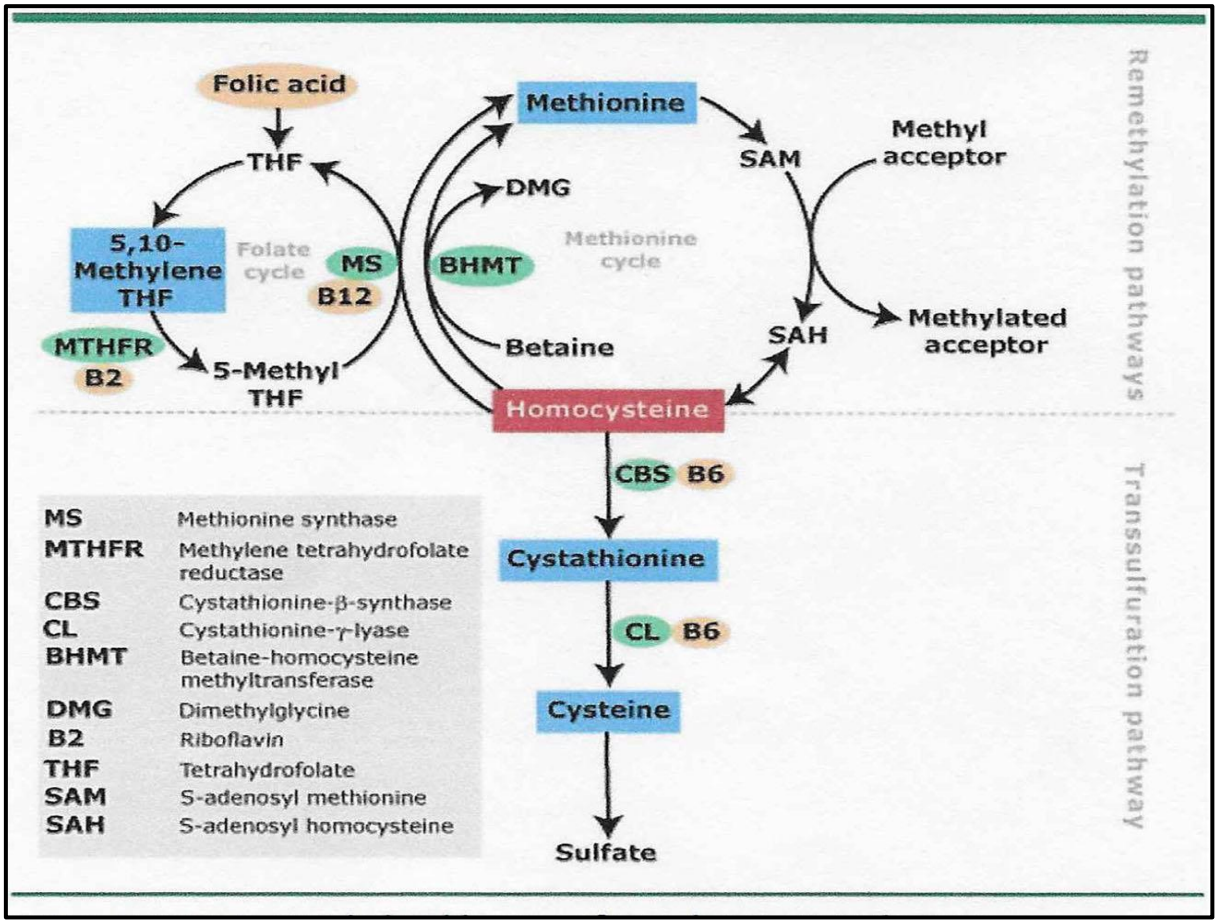

Figure 5: Pathways of homocysteine metabolism. Homocysteine is metabolized by one of two divergent pathways: transsulfuration and remethylation (Kang and Rosenson, 2018)

I.2.3.3 Etiologic mechanisms of Hcy in vascular disease

Experiments suggest that Hcy promotes aggregation of platelets; endothelial damage mediated through H_2O_2 production also Hcy increases DNA synthesis, growth, and cyclin A gene expression. Hcy rapidly reacts with endothelium-derived relaxing factor/nitric oxide (NO) to form Snitroso- Hcy, which acts as a potent antiplatelet agent and vasodilator. The formation of this adduct may attenuate H_2O_2 production from Hcy and there by protect against the atherogenic properties of Hcy. According to this model, vascular injury is caused by an imbalance between NO production from dysfunctional endothelial cells and the levels of Hcy (Refsum et al., 1998).

I.3 Hyperhomocysteinemia

I.3.1 Definition of Hyperhomocysteinemia

Hyperhomocysteinemia is usually defined as an elevation of plasma tHcy >15 µmol/l and may be caused by genetic defects, renal insufficiency, some drugs (Lentz, 2001 and Ganguly and Alam, 2015) or nutritional deficiencies of folate, vitamin B6, or vitamin B12 (Bellamy et al., 1998 and Lentz, 2001).

I.3.2 Hyperhomocycteinemia and cardiovascular disease

Even a mild elevation of plasma tHcy to levels within the high-to-normal range (10 to 15 µmol/L) may increase cardiovascular risk (Lentz, 2001). Hyperhomocysteinemia is an important risk factor for premature cardiovascular disease (Willems et al., 2002 and Eloudi et *al*., 2011). Levels of hyperhomocysteinemia have been classified into mild moderate (homocysteine level, 16 to 30 µmol per liter), intermediate (>31 to 100 µmol per liter), and severe (>100 µmol per liter). Meta-analysis calculated an odds ratio of 1.32 (95% confidence interval 1.19 to 1.45) for the presence of ischemic heart disease with a 5 µmol/l increase in serum homocysteine (Melhem et *al*., 2003). Actually, factors indicates that hyperhomocysteinaemia (Hcy level > 15µmol/L) is an important independent risk factor of atherosclerosis and thrombotic disease (Eloudi et *al*., 2011). Studies *in vitro* have demonstrated that homocysteine may injure endothelium this endothelial injury appears to be an early event in the promotion of atherogenesis and may be one mechanism whereby homocysteine leads to an increased risk of both arterial and venous disease (Bellamy et al., 1998).

Endothelial dysfunction is a key mechanism in the current hypothesis of atherothrombosis, and it is shown that functional impairment of the endothelial function, defined as an impairment of endothelium-dependent coronary blood flow, precedes significant arterial vessel disease (Willems et al., 2002),

Homocysteine may promote the oxidation of low density lipoprotein cholesterol, vascular smooth muscle cell proliferation, platelet activation and clotting factors and endothelial dysfunction. As a result, homocysteine metabolism abnormalities are now receiving increasing attention because of their potential role in the pathogenesis of atherosclerosis and other diseases such as venous thrombosis (Booth et al. 2000).

I.3.3 Oxidative stress and hyperhomocysteinemia

Homocysteine has a pro-oxidative action *in vitro,* its thiol group being oxidized to form reactive oxygen species that are responsible for cell damage and dysfunction (Outinen et *al*., 1999).

In eukaryotic cells, the endoplasmic reticulum (ER) is the main site for the processing of transmembrane, secretory, and resident proteins. To ensure correct turnover of newly synthesized proteins, ER contains many chaperone molecules such as GRP78 (glucose regulated protein-78), PGP-49, calnexin, calreticulin and bisulfide isomerase protein. Proteins with misfolding or non-refolding will be degraded by the cytoplasmic proteasome. Endoplasmic reticulum stress is a pathological condition where "misfolded" or unfolded protein accumulates (Oz et *al*., 2005). ER stress activates the unfloded. The pro-oxidant effect of L-homocysteine can be explained by a mechanism called "eNOS uncoupling". Briefly, L-homocysteine causes eNOS to become the major source of the superoxide anion (O^{-2}). O^{-2} rapidly inactivates NO, leading to the formation of high concentration of peroxynitrite (ONOO-) a very potent and toxic oxidant. Another potential mechanism for superoxide (O^{-2}) anion accumulation in the presence of Hcy is the inhibition of animal-induced NO production with Hcy, it has been shown a plasma elevation of asymmetric dimethylarginine (ADMA). Another possible mechanism of Hcy-induced oxidative stress is the disruption of the function of two antioxidant enzymes: superoxide dismutase (SOD) and peroxidase glutathione (GPX). SOD is an enzyme catalyzing the reduction of superoxide anion (O^{-2}) to hydrogen peroxide. Plasma Hcy level is reported to be correlated with extracellular SOD increase in patients with homocystinuria and in patients with moderate hyperhomocysteinemia. This effect is probably caused by the decrease in extracellular SOD binding to the endothelial cell surface, because Hcy could alter the surface of the heparan sulfate proteoglycan. This effect could lead to a loss of its ability to protect the endothelial surface against oxidative stress. Research show that Hcy decreases the expression and activity of glutathione peroxidase (GPX) (Figure 5) (Au-Yeung et *al*., 2004). GP_X catalyzes the reduction of hydrogen peroxide (H_2O_2) in H_2O by oxidation of GSH to GSSG. Hyperhomocysteinemia may prevent and limit the antioxidant potential of cells. This has been demonstrated in genetically modified animal models. GP_X overexpression has been reported to attenuate homocysteine-induced endothelial dysfunction. When the endothelium is exposed to Hcy

or methionine, the concentration of ADMA in the culture medium increases in a dose and time dependent manner and is correlated with a decrease in NO synthesis (Collins and Cybulsky, 2001).

Homocysteine might directly or indirectly lead to oxidative stress via the pathways shown in the figure 6 (Sharma et al., 2006). The toxic effects of homocysteine are its ability to generate reactive oxygen species there by producing oxidative stress. It is generally proposed that homocysteine, due to the presence of a thiol group, can rapidly auto-oxidize in circulation in the presence of ceruloplasmin, the major copper binding protein in plasma, to form homocystine and hydrogen peroxide (H_2O_2), thereby generating oxidative stress (Jacobsen, 2000 and Sharma et al., 2006).

However, several recent reports indicate that transition metal catalyzed oxidation of homocysteine is a complicate process. In fact transition metal catalyzed oxidation of cysteine has been reported to be much faster than that of homocysteine and although the concentration of cysteine is about 20–25 times higher than that of homocysteine it is usually not considered a risk factor for cardiovascular diseases. Therefore, it seems unlikely that the deleterious effect of homocysteine is due to the generation of hydrogen peroxide via metal catalyzed auto-oxidation. However, homocysteine might indirectly result in oxidative stress by decreasing the transcription, translation and catalytic activity of antioxidant enzymes like glutathione peroxidase (GPx) and superoxide dismutase (SOD) (Sharma et *al*., 2006).

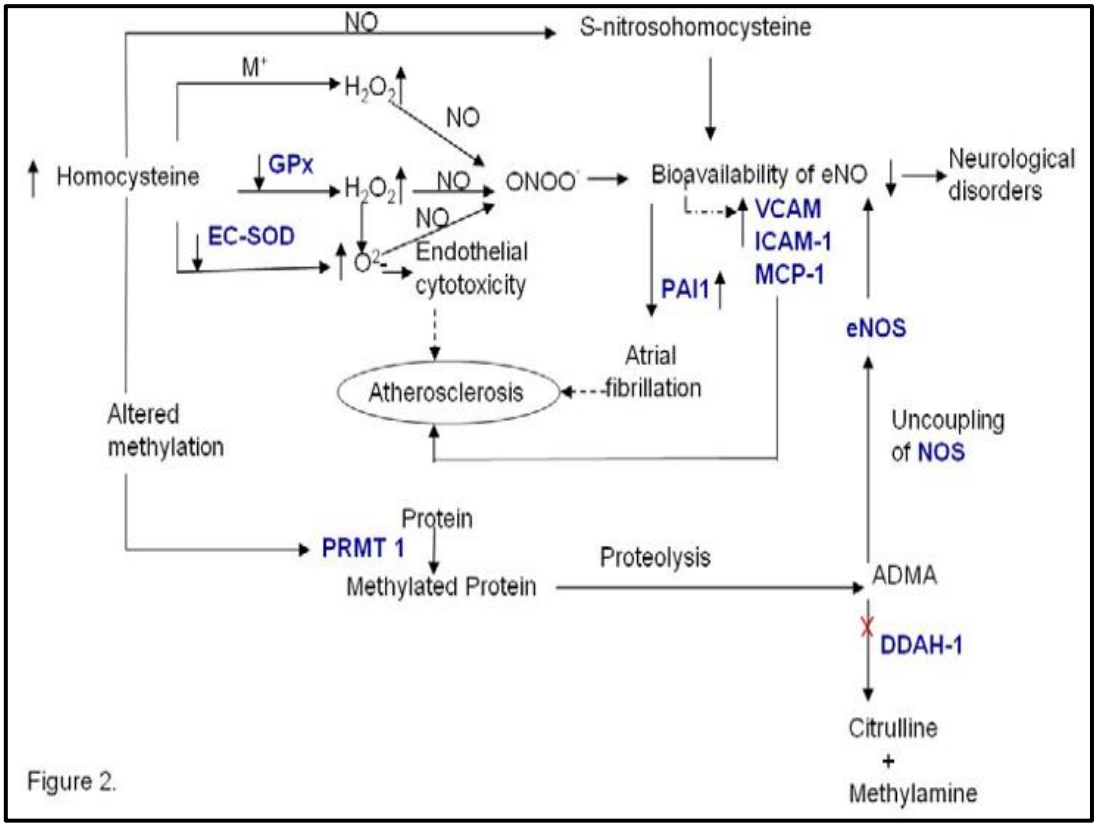

Figure 6: Hyperhomocysteinemia and Oxidative Stress (Sharma et al., 2006).

I.4 Cancer

I.4.1 Definition of cancer

Cancer is a disease of inherited or somatic alterations in genes, are what make a normal cell ignore growth controlling signals and form a tumor that eventually leads to the destruction of the organism (Shipitsin and Kornelia, 2008).

I.4.2 Appoptosis

Apoptosis is one of the most potent defenses against cancer, since this process eliminates potentially deleterious, mutated cells (Lockshin and Zakeri, 2004). Apoptosis include both intra- and extracellular stimuli, such as DNA damage, disruption of the cell cycle, hypoxia, detachment of cells from their surrounding tissue, and loss of trophic signaling Apoptosis occurs primarily through two well-recognized pathways in cells (Danial and Korsmeyer, 2004). Both effector mechanisms of apoptosis are associated with caspase activation and include the intrinsic, or mitochondrial-mediated, effector mechanism and the extrinsic, or death receptor–mediated, effector mechanism (Reed,

2004). In addition to mitochondria, other organelles, including the endoplasmic reticulum, Golgi apparatus, and lysosomes, may also contribute to damage sensing, pro-apoptotic signaling, and caspase activation (Sun et al., 2004 and Jaattela, 2004). The endoplasmic reticulum, as an important apoptotic control point, displays anti-apoptotic Bcl-2 and proapoptotic Bax and Bak proteins (Danial and Korsmeyer, 2004). The intrinsic pathway of apoptosis relies primarily on the permeabilization of mitochondrial membranes, with associated release of apoptogenic mitochondrial proteins, leading to activation of caspase 9 and downstream cleavage of caspases 3, 6, or 7. A third pathway involving granzyme has been identified, one that directly activates caspase 3. A critical element and commonality among these pathways is the involvement of caspases and, specifically, the activation ofcaspase 3, the pivotal committed executioner caspase of apoptosis (Keith,2012).

I.4.3 Network of cancer and oxidative stress

Some of the ways in which reactive species could facilitate cancer development are mentioned in the figure 7, when:

1. Direct oxidative damage to desoxyribonucleic acid (DNA).

2. Aldehyde end-products of lipid peroxidation (e.g. malondialdehyde and 4-Hydroxynonenal 4-HNE) form mutagenic adducts with DNA bases. Radicals ($RO^{\cdot}$ and $RO_2^{\cdot}$) formed during lipid peroxidation might also attack DNA, e.g. Hydroxyl radical attack upon DNA generates multiple mutagenic purine, pyrimidine and deoxyribose oxidation products.

3. Reactive species (RS) can help to convert pro-carcinogens into ultimate carcinogens, some of which then lead to more RS formation.

4. Reactive species (RS) can damage proteins, e.g. chromatin proteins, DNA-repair enzymes and DNA polymerases (perhaps increasing the error rate of replication).

5. 4-Hydroxynonenal (4-HNE) can be pro proliferative and is a powerful redox modulator and some other products of lipid peroxidation may act similarly. Lower panel: how RS-induced changes in DNA can cause mutations (Halliwell, 2007).

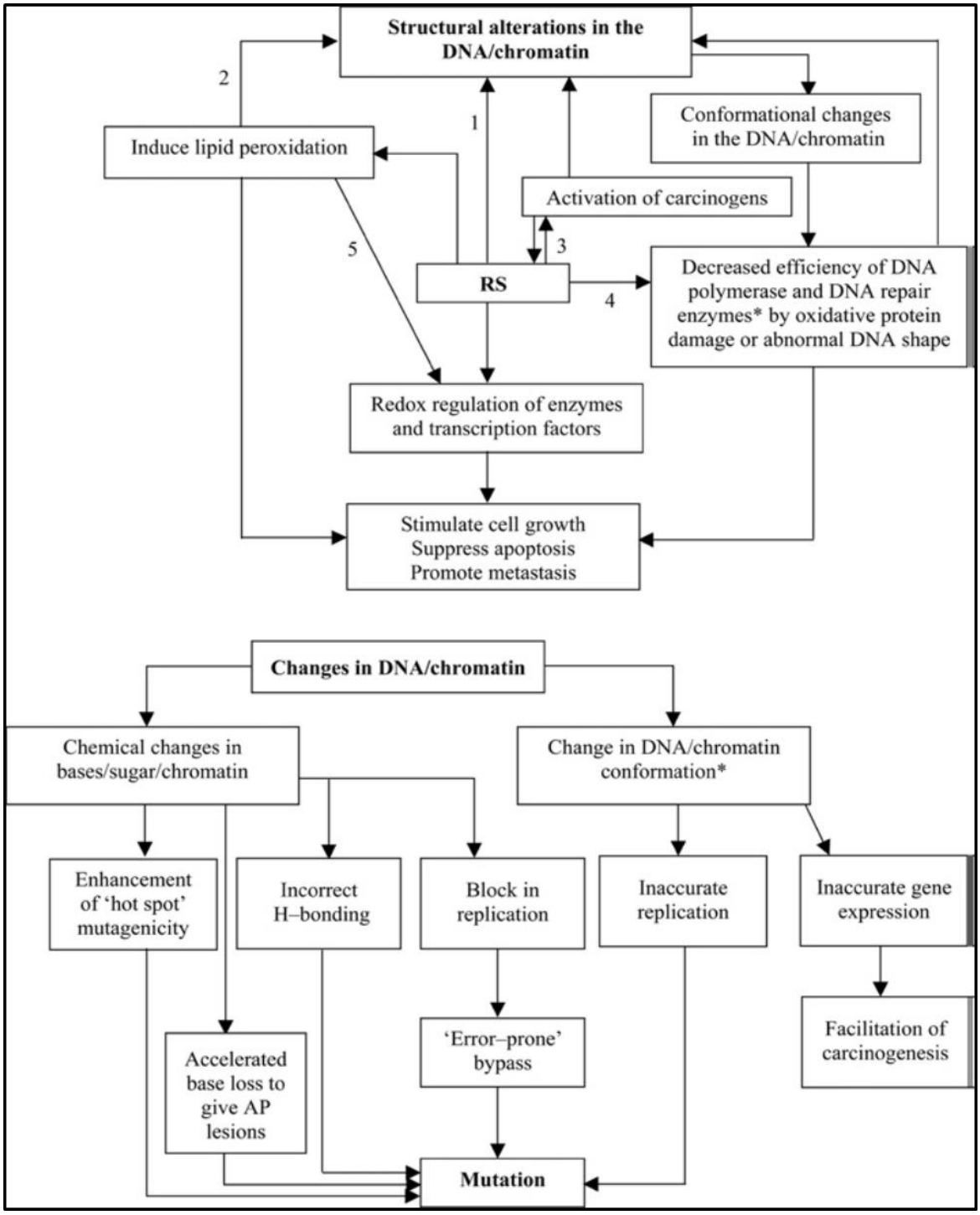

Figure 7: The ways in which reactive species could facilitate cancer development (Halliwell, 2007).

I.5 *Vitis vinifera*

I.5.1 Definition

The medicinal plant *Vitis vinifera* L. (Vitaceae), Turkish name, "Asma", is a perennial woody vine, usually climbing by tendrils, native to Asia Minor and then introduced in Europe and other continents (Deliorman et al., 2007). The medicinal and nutritional value of grapes *(Vitis vinifera*) has been indicated for thousands of years. Egyptians consumed this fruit at least 6,000 years ago and several ancient Greek philosophers praised the healing power of grapes (Busserolles et al., 2006).

Table 1: Classification of *Vitis Vinifera* (Andrasovsky, 1933)

Domain	Eukaryotic
Kingdom	Plantae
Phylum	Angiospermae
Class	Magnoliopsida
Order	Rhamnales
Family	Vitaceae
Genus	*Vitis*
Species	*Vitis vinifera*

Figure 08: Leaves of *Vitis vinifera*

I.5.2 Statistical situation of the world grape-vine sector

The Vine is one of the most cultivated fruit species in the world. The world's vineyards cover five continents and cover an area of 8 million hectares (OIV report: International Organisation of Vine and Wine, 2018). The majority of the world's wine-growing areas are located in Europe (59%), the rest being spread over Asia (22%), America (12.5%), Africa (4.9%) and Oceania (0.7%). *Vitis vinifera L.* is a widespread crop in Algeria. In 2000, following the Algerian agriculture ministry, vineyards occupied an area close to 56,500 ha. The world's annual wine production is estimated at 287.3 millions hectoliltres. Europe, with about 74% of this production, occupies a dominant position.

I.5.3 Vinery by-products

Vinery by-products can be classified into three categories: marc and its derivatives (pulp and seeds), leaves and branches. The mass of by-product left from the vine is large but their composition is poorly known and highly variable, resulting in poor use and is intended for animal feed (Pheleps, 1987).

I.5.4 Therapeutic properties

Vitis (*V.*) *vinifera*, is an Asian native perennial woody vine. From different parts of this plant essentially fruits, several preparations used in folk medicine have been derived (Bombardelli and Morazzonni, 1995). In Ethnopharmacology, the infusion of the leaves of red varieties has been used as haemostatic and for diarrhea treatment. Fresh leaves have been used externally to heal wounds and to lance abscesses (Baytop, 1999). Grape leaf based medicines are traditionally used for diarrhea, hepatitis and stomachaches (Kapoor, 1990, Bombardelli and Morazzonni, 1995, Felicio et al., 2001 and Kallel et al., 2008). Grapes, seeds, and leaves have been used for preventing heart and blood vessels diseases, varicose veins, hemorrhoids, “hardening of the arteries” (atherosclerosis), high blood pressure, swelling after injury or surgery, heart attack and stroke.

Moreover, grape leaf has been used for attention deficit-hyperactivity disorder (ADHD), chronic fatiguesyndrome (CFS), diarrhea, heavy menstrual bleeding, uterine bleeding, andcanker sores. It has been also used as a mild laxative forconstipation (Kapoor, 1990 and Hebash et al., 1991).

Chapter II :
Material and Methods

II.1 The Effects of grape Leaves Extract on hyperhomocysteinemia induced inflammatory endothelial damage in cardiovascular diseases

II.1.1 Plant material

The plant material was collected in August, fully mature and untreated with pesticide grape (*Vitis vinifera* L.) leaves from the Medea area-Algeria. Leaves were rinsed in tap water and freeze-dried. Afterwards, the freeze-dried leaves were crushed with a blender for 5 min and the resulting powder was collected and stored in the dark at 5 °C in a sterile bag and under vacuum for further use (figure 09-10).

Figure 09: Dried *Vitis Vinifera* leaves

Figure 10: Powdered *Vitis vinifera* leaves

II.1.2 Chemicals

All the used solvents and the formic acid of HPLC-grade (from Merck, Darmstadt, Germany). The water was purified by a milli-Qplus system from Millipore (Milford, MA, USA). All the employed Reagents were of analytical grade, purchased from Carlo Erba (Milan, Italy). The standards of HPLC-grade[malvidin-3-glucoside (n° 04288)], the 5,5′-dithiobis-(2 nitrobenzoic acid), 5,5′-dithiobis-(2 nitrobenzoic acid) (DTNB) and all the rest of chemicals were purchased from Sigma-Aldrich, Inc. 0.45 µm Polytetrafluoroethylene (PTFE) syringe membrane filters was purchased From Waters Co. - Milford, MA, USA.

II.1.3 Methods

II.1.3.1 Effect of grape leaves on the inflammation induced by Hyperhomocysteinemia

II.1.3.1.1 Animals and Diets

Twenty eight Albino *Mus musculus mice*, (2 to 2.5) months older, weighed between (18 -27 g), were used in this experiment. They were provided by the central pharmacy Constantine (Algeria). The mice were separated into four groups in four cages according to their body weight. The planned diet was given in the form of balls prepared with 0.5 mg of white flour and distilled water for 15 days. The first group (F) treated with white flour (0.5 mg / kg / day), second group (M) was administered with with L-methionine (1g / kg / day), third group (MP) with (L-methionine + *Vitis vinifera*) (1g /kg + 500 mg / kg / day). The fourth group (P) was administered only with *Vitis vinifera* leaves (500 mg / kg / day). Mice were housed at normal conditions of the animal house throughout the treatment period.

II.1.3.1.2 Blood analysis

At the end of experiment, mice were fasted overnight, and the blood samples were collected from the retro orbital vein into EDTA tubes by using glass capillaries. They were centrifuged immediately, and the plasma was stored at -30°C. The values of plasma hs-CRP were measured by the immunoturbidimetric method on a Cobas integra 400 plus analyzer (Roche).Total homocysteine (t-Hcy) was estimated by competitive solid phase chemiluminescence immunoassay (Ibn Sina Clinic).

II.1.3.1.3 GSH Glutathione assay (GSH)

After sacrificing the animals, the liver was dissected and washed with NaCl 0.9%. Then, the homogenate had been prepared with 0.5g of the liver homogenized in 2 ml of TBS (Tris50 mM, NaCl 150 mM, pH 7.4). Next, it was centrifuged at 9000 g for 15 min at 4˚C. After that, the supernatant used for the determination of glutathione reduced (GSH), then it was measured spectrophotometrically by using 5,5′-dithiobis-(2 nitrobenzoic acid) (DTNB) as a coloring reagent, following the method of Weckbekeret al.,1988 (Rahman et al., 2006).

II.1.3.1.4 Histology

At the end of the *in vivo* study, the animals were sacrificed and the aorta was taken.

1) Dissection protocol

- We pin the animal down with the belly facing up. Then, we wet it down with ethanol by washing the carcass in order to disinfect the tissues.
- Using scissors, we cut along the ventral midline from the groin to the chin, being careful to only cut the skin and not the muscle wall underneath.
- We make an incision from the start of the first incision downward.
- After opening both the abdominal cavity and the rib cage, the organs were collected and cleaned with phosphate buffer saline (NaCl) pH 7.4.

2) Histological sections preparation

After the dissection of the animals, the aortas were cut and kept in small containers filled with diluted formol 10%. The first step in protocol is to incubate the organs in the Formol (2%).

3) Tissue fixation

As a second step, the dehydration is performed through a series of ethanol solution (50%, 70%, and 96%), each step was placed for approximately 30 min (3×30 min=1h

30min). The organs were then kept in small containers filled with butanol for an entire week. After that, they were immersed twice in a xylene solution, for 10 min each time.

4) Infiltration and embedding in paraffin and Hematoxylin eosin staining

The organs were immersed in paraffin twice, for 2 hours each time. The sectioning is performed with a microtome. Paraffin slices of 5µm thick were stained following the haematoxylin eosin staining protocol.

II.1.3.2 Analysis of anthocyanins and non-anthocyanin phenolic compounds of GLAV by reverse phase liquid chromatography-diode array detection/electrospray mass spectrometry (RPLC-DAD/ESI-MS)

II.1.3.2.1 Sample preparation

Three grams of powdered leaves were extracted three times at 1:2 (weight/volume) (w/v) ratios with cold methanol: HCl (1000:1 (volume/volume) (v/v) by using an ultra turrax (Ultra Turrax-Tube Drive, BM-G-ball-mill tube, IKA, Germany) with 10/CS glass balls, for 3 min of each sample. After the extraction, centrifugation took place (1600 g, 15 min, and 4 °C), the supernatant was collected and stored on ice. The pellet was re-subjected to subsequent extraction, and a final volume of 14.5 ml was collected. The methanol/HCl extract was first filtered through the 0.45 µm membrane PTFE, and then it was exposed to Speed Vac concentration (SC250P1-250, Thermo Fisher Scientific Inc, Waltham, MA, USA) at 20 °C until dryness. Next, the residue was brought to a final volume of 10 ml by adding formic-acidified (pH 3.2) MQ water and kept at -20 °C until analysis. Discarding the pellets, the extraction was performed in triplicate (Kammerer et al., 2004).

II.1.3.2.2 HPLC-DAD-ESI-MS characteristics and protocols

1) HPLC-DAD and HPLC-MS apparatus

The reverse phase liquid chromatography (RPLC) was performed with a High Pressure Liquid Chromatography system consisting of Hewlett-Packard series 1100 L equipped with a Diode Array Detector (DAD) operated by a HP 9000 workstation (Agilent Technologies, Palo Alto, CA, USA). The HPLC-MS system was equiped with HPLC-DAD instrument coupled to a quadripole mass spectrometer HP 1100 MSD electrospray

interface (ESI) (Agilent Technologies, Palo Alto, CA, USA). The Separation had been occurred on a reverse-phase Waters Nova-Pak C_{18} column [150 mm x 3.9 mm, 4 µm] for anthocyanins analysis and a reverse-phase Waters Nova-Pak C_{18} column [300 mm x 3.9 mm, 4 µm] for non-anthocyanin phenols, and both had been kept at 26°C with a pre-column of the same phase (Villiers et al., 2004 and Kammerer et al., 2004).

2) DAD and ESI-MS parameters

- The non-anthocyanin phenols DAD was performed from 220 to 380 nm and ESI-MS parameters were: drying gas (N_2) at 350°C with a 10 L/min flow, nebulizer pressure at 380 Pa (55 psi), and capillary voltage of 4000 V. The ESI scanned the mass from m/z 100 to 3000, employing a fragmentator voltage gradient of 100 V from 0 to 200 m/z and 200 V from 200 to 3000 min (Dobes et al., 2013).

- On the other hand, the anthocyanins DAD was performed from 260 to 600 nm, and ESI-MS parameters were: drying gas (N_2) at 350°C with a 10 L/min flow, nebulizer pressure at 380 Pa (55 psi), and capillary voltage of 4000 V. The ESI scanned the mass from m/z 100 to 1500, employing a fragmentator voltage gradient of 100 V from 0 to 17 m/z and 120 V from 17 to 55 min (Dobes et al., 2013).

3) Elution parameters

1. The elution of non-anthocyanin phenols was performed at a 0.7 ml/min gradient flow of solvent 'A' and 'B'. The former was a combination of water/acetic acid (98:2, v/v), and the latter constitued of water/acetonitrile/acetic acid (78:20:2, v/v/v). A linear gradient started with 0% of 'B' and 100% of 'A', and then reached 80% of 'B' and 20% of 'A' after 55 min. After that, slight linear increase of 'B', from 80 to 90%, between 55 to 57 min, was observed, and then it remained isocratic at 90% of 'B' and 10% of 'A'. Next, the process increased linearly from 90 to 95% of 'B' during 10 min. Finally, and still in a linear way, it reached 100% of 'B' at 90 min. The column was washed with Methanoland re-equilibrated from 90 to 120 min. The volume of the injected leaves extract was 15 µL.

2. Eluent of anthocyanins was performed at a 0.8 ml/min gradient flow of solvent 'A' and 'B'. The former was a combination of water/formic acid (90:10, v/v), and the latter constituted of water/methanol/formic acid (45:45:10, v/v/v). A linear

gradient started with 15% of 'B' and 85% of 'A', then it reached 80% of 'B' and 20% of 'A' after 30 min. After that, the process continued in an isocratic way, from 30 to 45 min, with 80% of 'B'. The column was then rinsed with methanol and re-equilibrated from 43 to 75 min. The injected volume of leaves extract was150 µL, and formic acid was employed as a pH modulating agent in order to optimise the anthocyanins detection by maximizing the absorption in the λ 520 nm region.

4) HPLC/DAD/ESI-MS identification of anthocyanins

The identification of malvidin 3-glucoside and cyanidin 3-glucoside was performed by comparing the results of commercial standards (Sigma-Aldrich) with the positive ion mass spectra achieved from the ESI-MS (retention time (tR), UV λmax, and MSn) of the leaves extract, while other anthocyanins were identified by comparing the ESI-MS attained results against the ones available in the literature (Table 4).The flavonols were identified (glucose/galactose and glucuronide derivatives) under ESI-MS. This latter was achieved according to the molecular and fragment ions [M-H-162]- (quercetin-3-galactoside/glucoside) and [M-H-176]- (quercetin-3-glucuronide), (Figures 28 and 29). In addition, the identity of all other constituents was validated by comparing the attained retention times (tR), UV λmax, and MSn of peaks from the leaves extract with those reported in the literature.

5) Compound quantitative analysis by HPLC/DAD

1. The quantification of anthocyanins in the leaves extract was obtained by measuring peak areas at 530 nm and taking into account the external standard calibration curve of malvidin-3-glucoside, which was measured at 524 nm. The concentrations were expressed as 'µg malvidin-3-glucoside equivalents/g of freeze-dried leaves'. The contribution of single anthocyanins was calculated and expressed in % of the total anthocyanins content.

2. The quantification of flavonols was performed according to the peaks attained at 340 nm and calculating the concentrations as 'µg quercetin or kaempherol equivalents/g of freeze-dried leaves' according to external standard calibration

curve of quercetin and kaempherol. The contribution of single flavonols was calculated and expressed as % of the total flavonol content.

3. Quantification of *trans*-caftaric acid was based on an external standard calibration curve carried out at 340 nm and expressed as 'µg trans-caftaric acid /g of freeze-dried leaves'.

4. The external standard calibration curves for malvidin-3-glucoside, quercetin, kaempherol and trans-caftaric acid, which were performed in duplicate by using five dilutions within linearity and an R^2 values, were 0.98, 0.97, 0.98 and 0.99 respectively.

II.1.3.5 Statistical analysis

Statistical analysis was carried out by one–way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests using statistics software package (SPSS for Windows, 20.0, Chicago, USA). P values < 0.05 were considered as statistically significant.

II.2 Phenols from Grape Leaves Counteract Cell proliferation and Modulate Apoptosis-Related Gene Expression in MCF-7 and HepG2 human Cancer Cell Lines

II.2.1 Chemicals and cells

All solvents used were HPLC (High Performance Liquid Chromatography) grade purified (Merck, Darmstadt, Germany); water was purified using a milli-Qplus system from Millipore (Milford, MA, USA). Reagents employed were of analytical grade; Folin-Ciocalteu reagent and Sodium Carbonate (Na_2CO_3) were purchased from Carlo Erba (Milan, Italy); DPPH (2,2-diphenyl-1-picryhydrazyl) and gallic acid (3,4,5-trihydroxybenzoic acid) were purchased from Sigma-Aldrich, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) spin trap and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Milan, Italy).

Hepatocarcinoma cells (HepG2) and breast cancer cells (MCF-7) were obtained from the anatomic pathological civil hospital of Cagliari, Italy. HUVECs cells were obtained from Gibco™. Cisplatin was obtained from the oncological service hospital of Sassari, Italy.

Dulbecco's phosphate buffered saline (DPBS) was purchased from Euroclone (Milano, Italy); Dulbecco's modified Eagle's Medium with phenol red (DMEM) and fetal bovine serum (FBS) from Life Technologies (Grand Island, NY, USA); Medium 200 and LSGS (5-003-10) from Gibco™. TRIzol reagent, SuperScript® VILO™ cDNA Synthesis Kit, Platinum Quantitative PCR Supermix UDG Kit, SybrGreen I, primer and fluorescein from Life Technologies (Grand Island, NY, USA). L-glutamine, Penicillin, Streptomycin, nonessential amino acids from Euroclone (Milano, Italy). (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction MTT Cell Proliferation Assay ATCC® 30-1010K kit was purchased from Invitrogen Co. All primers are representing in the table 2.

Table 2: Primers sequences used for real-time PCR reactions.

Primers	Forward	Reverse
HGAPDH	GAGTCAACGGATTTGGTCGT	GACAAGCTTCCCGTTCTCAG
BAX	TCTGACGGCAACTTCAACTG	TTGAGGAGTCTCACCCAACC
BCL-2	GGATTGTGGCCTTCTTTGA	ACAGTTCCACAAAGGCATCC

II.2.2 Extraction procedure

The accelerated solvent extraction (ASE) was performed on a Dionex ASE 350 (Dionex Thermo Fisher Scientific Inc. in the United States). Powdered leaves (1 g) were placed between two layers of glass bead in 22 ml Dionex (ASE 350) stainless-steel cell. The cells were equipped with a stainless steel fit and a cellulose filter.

Table 3: Conditions of ASE extraction procedure.

Temperature (°C)	40
Pressure (PSI)	1500
Number of Cycle	2
Extraction time of one cycle (minutes)	5
Concentration of Ethanol (%)	60 Ethanol /40 water
Type of water used	Ultrapure

The extraction cell was arranged in the cell tray and was extracted using the conditions presented in Table 3. The automated extraction cycle was as follows: the cell containing the sample was prefilled with the extraction solvent, pressurized (1500 psi), then heated for 5 minutes followed by a static period of 5 minutes and two cycles extraction were occurred for ten minutes. The sample was extracted with two solvents ethanol/ water or only water at the fixed temperature during 5 minutes per cycle. Then, the cell was rinsed with fresh extraction solvent (60% of the extraction cell volume) and purged with a flow of nitrogen (150 psi during 90 s). Extracts (34 ml) were collected into 60 ml glass vials. The extraction was done in quadruplicate for each variety. The ethanolic extract was evaporated under a nitrogen flow to avoid oxidation processes then, the two extracts were frozen at - 20 °C and lyophilized. Powder obtained after ice-draying obtained was weighted and collected in sterile vial kept stored at -80°C. Accelerated solvent extraction performed with the lowest extraction temperature to avoid the maximum degradation of thermolabile compounds.

The extraction yield was calculated as follows:

$$\text{Yield } \% = \frac{(\text{the weight of freeze} - \text{dried recover})}{1 \text{ gram (initial weight of leaf powder used)}} \times 100$$

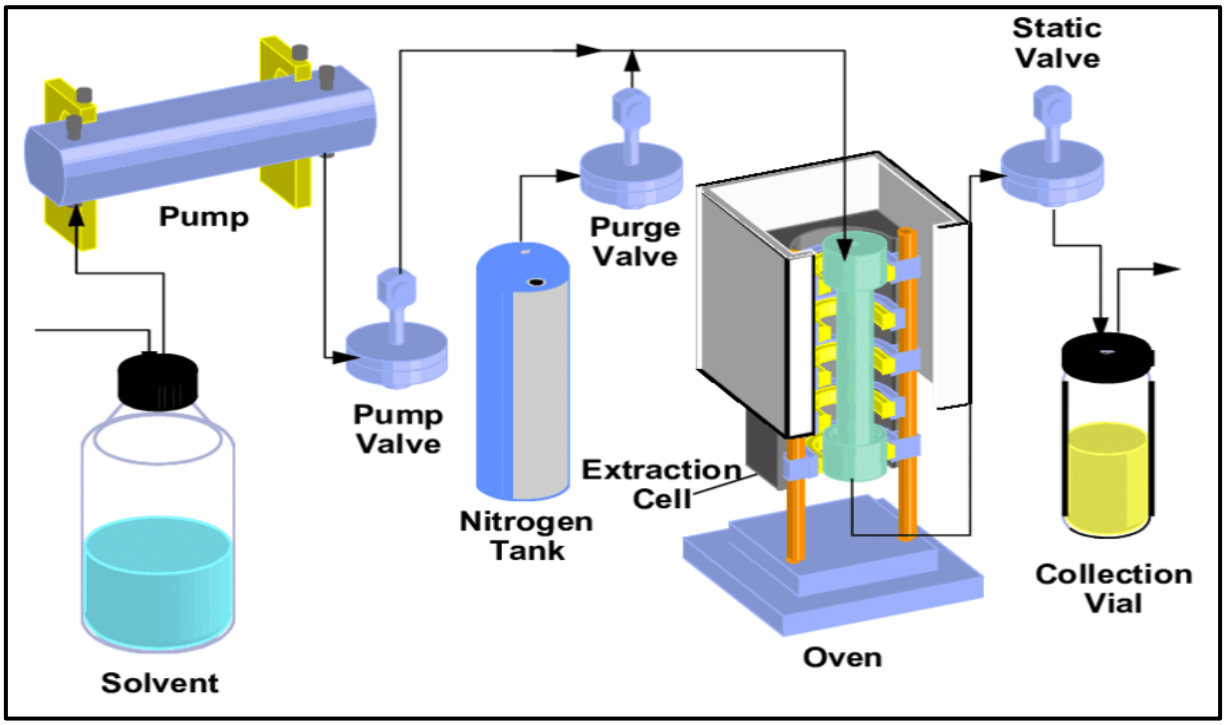

Figure 11: Flow diagram of the Accelerated Solvent Extractor 200ä (from: Dionex GmbH, Idstein, Germany) (Van der et al., 1997)

II.2.3 Total phenolic (TP) content

The total phenolic content was measured using the modified Folin–Ciocalteau method (Folin and Denis, 1912, Folin and Ciocalteu, 1927 and Singleton and Rossi, 1965).1 mg of each lyophilized extract placed into a 15 ml centrifuge tube (Corning, U.K. code 430053) and 9 ml of cold ethanol (80%) (1:10 w:v) were added, then vortexed (Stuart, U.K. model SA8.) at 1600 rpm for 2 minutes and centrifuged (ALC-Centrifuge 4227R, Milan-Italy) at 16.000 g at 4 °C for 15 minutes. Then, the mixture was kept on ice until use. 200 µl of each extract was mixed with 1 ml of the Folin–Ciocalteu reagent and allowed to react for eight minutes. Then, 800 µl of sodium carbonate solution (0.075 Na_2CO_3 ml^{-1}) were added to the mixture which was incubated in the dark for one hour at room temperature (20±3°C) followed by an additional hour incubation at 0 °C. The absorbance was read at 760 nm with a spectrophotometer (Agilent 8453).

Results were expressed as milligrams of Gallic acid Equivalent/g of dry weight. Calibration curve of Gallic acid was prepared with five points from 50 to 500 mg/L with $R^2 = 0.996$.

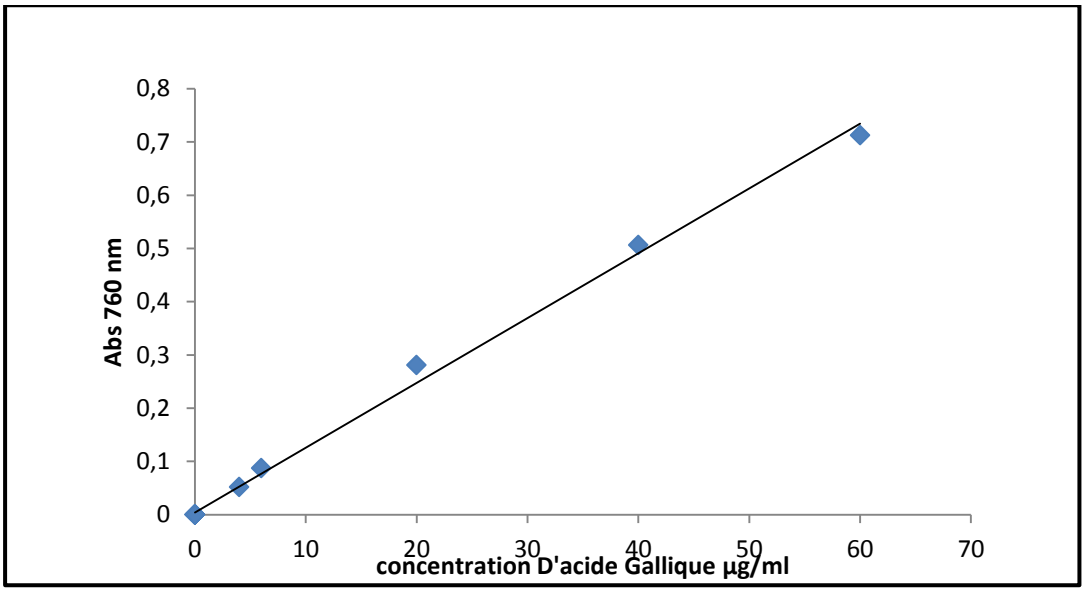

Figure 12: Calibration curve of Gallic acid

II.2.4 Antioxidant activity

II.2.4.1 Spin trapping assay of the ˙OH radical

The hydroxyl radical scavenging activity was determined with the spin trapping method coupled with Electron Paramagnetic Resonance spectroscopy according to Fadda et al., (2018). The hydroxyl radicals were generated by the Fenton reaction and trapped with a nitrone spin trap 5,5-dimethyl-pyrroline N-oxide (DMPO) (Fadda et al., 2015). 20 mg of the freeze-dried extract were mixed with 1 ml of ultrapure water degassed under nitrogen flow which was prepared as stock solution. Serial dilutions were prepared from the stock solution and depending on the results; the correct concentration for each extract was established. 100 µl of the diluted samples were mixed with Fe (II) sulfate 0.1 mM (100 µL), 112 µL DMPO 26 mM (112 µL) and H_2O_2 1 mM (100 µL).

The DMPO-OH adduct was detected with a Bruker EMX EPR spectrometer operating at the X-band (9.4 GHz) using a Bruker Aqua-X capillary cell. EPR instrument was set under the following conditions: modulation frequency, 100 kHz; modulation amplitude, 1 G; receiver gain, 1×10^5; microwave power, 20 mW. EPR spectra were recorded at room temperature immediately after the preparation of the reaction mixture. The concentration of the spin adduct DMPO-OH was estimated from the double integration of spectra. The hydroxyl radical scavenging activity was expressed as IC50 on the basis of the percentage of inhibition calculated as follows:

$$\% \text{ inhibition } = \frac{(A0 - As)}{A0} \times 100$$

Where A_0 is intensity of the spin adducts without extract and As is the absorbance of the adduct after the reaction with the extract. Different sample's concentrations were used to calculate the IC50 that is the extract concentration that halves the concentration of hydroxyl radical adduct of the blank. Three replications were performed for each dilution.

II.2.4.2 DPPH assay

The radical scavenging activity of ethanolic and water extracts of grape leaves was determined spectrophotometrically with the DPPH test (Choi et al., 2002).

Crude extract (30 µl of ASE ethanolic and water) at different concentrations (0.05, 0.1, 0.2 mg ml^{-1}) were mixed with 3 ml of a DPPH methanol solution (0.3 mM). A blank solution was prepared using methanol instead of the extract.

Solutions were stored in dark at room temperature for 30 minutes. The absorbance was measured at 518 nm and converted into the percentage of inhibition using the following equation:

$$\% \, inhibition = \frac{A0 - As}{A0} \times 100$$

Where A_0 is the absorbance of the sample without extract and A_S is the absorbance of the sample after the reaction with the extract. The DPPH radical scavenging activity was expressed as IC50. Three replications were performed for each dilution.

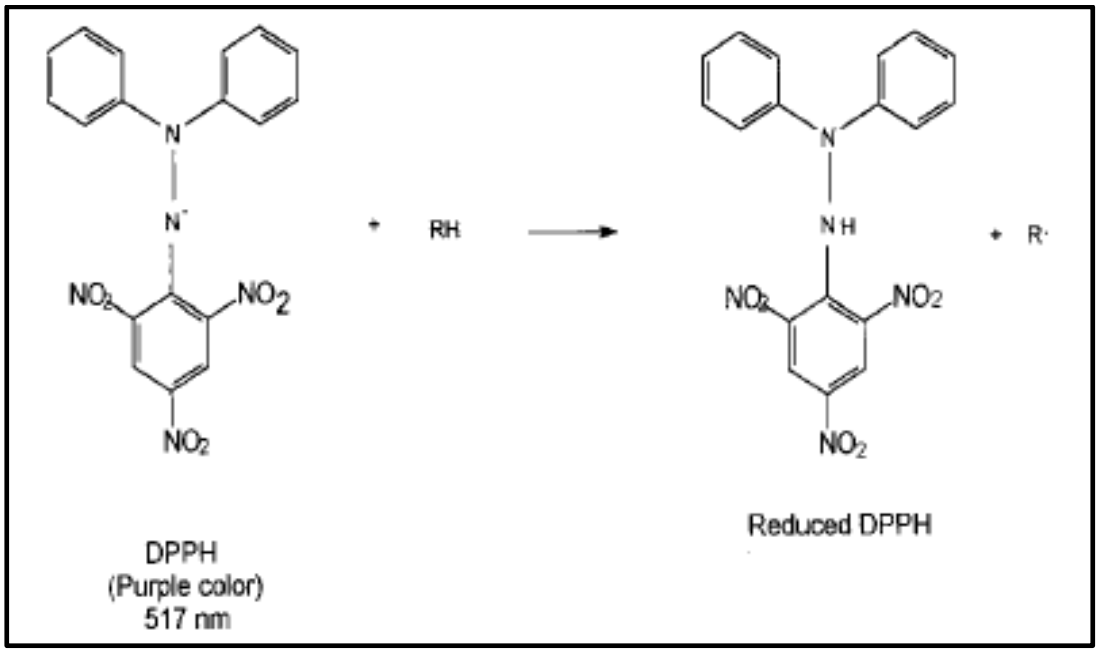

Figure 13: Scheme for scavenging of DPPH radical by an antioxidant (Santosh et al., 2002) / (RH is the antioxidant, R is the O-antioxidant)

II.2.5 Cell culture

HepG2 and MCF-7 cells were maintained in Dulbecco's modified Eagle's Medium with phenol red (DMEM), supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 200 of µM L-glutamine, 200 U/ml of penicillin, 10 µg/ml of streptomycin and 0.1 mM of non-essential amino acids. HUVEC cells were cultured in Medium 200 (Gibco™), containing LSGS (5-003-10; Gibco™), 200 U/ml of penicillin and 10 µg/ml of streptomycin. Cells were grown in 75 cm^2 tissue culture flasks in the culture incubator at 37 °C with 5 % CO_2 and saturated humidity.

II.2.6 Test of viability MTT assay

The anti-proliferative activity of ethanolic and water ASE extracts of *Vitis vinifera L.* leaves on HepG2, MCF-7 and HUVEC cells was determined using a cell viability test.

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] tetrazolium reduction assay is a colorimetric assay based on the ability of functional mitochondria to reduce by succinate dehydrogenase enzyme an insoluble formazan crystal which displays a purple color (Mosmann et al., 1983).

Then assesses the effects of the treatments studied on the overall growth of a particular cell population by determining the number of living cells remaining in the analyzed cell culture. After counting, HepG2, MCF-7 and HUVEC cells were seeded on a 96 well plate at concentration of 10.000/well in 200 µl and incubated at 37 °C in a 5 % CO_2 incubator (Thermo Fisher Scientific).

After 24 hours, medium was replaced with fresh medium containing compounds tested (Ethanol and water ASE crude extract) at concentration of 0,5 mg/ml, 1 mg/ml and 2 mg/ml. The negative control is performed in growing medium but positive control is prepared in medium supplemented with Cisplatin 10 µM. Every test was repeated three times. After one day, we substitute again medium with or without compounds and repeat the same treatment (treatment 2). The MTT substrate is prepared in a sterile Dulbecco's phosphate buffered saline (DPBS), then added to cells in culture at a final concentration of 650 µg/ml and incubated for 3 hours in the culture incubator at 37°C with 5% CO_2 and saturated humidity. After incubation, the medium was removed by aspiration and 200 µl/well Dimethylsufoxide (DMSO) was added to each well. Absorbance was read at 570 nm in a Gemini EMMicroplate Reader (Molecular devices). The percentage of cell proliferation was calculated relative to control wells designated as 100% viable cells using the following formula:

$$\frac{(\mathrm{At}-\mathrm{Ab})}{(Ac-Ab)}\times 100 = \%\, cell\ proliferation$$

Where At = Absorbance value of test compound (ASE extract), Ab = Absorbance value of blank (Medium alone), Ac = Absorbance value of control.

II.2.7 Gene expression

HepG2 and MCF-7 cells were plated into 24 well cell culture plates (60.000 cells/500µl for well) in culture medium for gene expression experiments to evaluate the anti-apoptotic activity of ethanolic and water ASE extracts of grape leaves. Extracts were prepared fresh just before each experiment and dissolved in DMEM.

After treatment, the total RNA was isolated using TRIzol reagent and quantified by measuring the absorbance at 260/280 nm (NanoDrop 2000, spectrophotometer Thermoscientific ND8008). Approximately 1 µg of total RNA was reverse-transcribed to

cDNA by SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies, Grand Island, NY, USA).

Quantitative polymerase chain reaction was run in triplicates using a CFX Thermal Cycler (Bio-Rad). 2 µl of cDNA were amplified in 25 µl reactions using Platinum Quantitative PCR Supermix UDG Kit. A Supermix 2X mixed whit Sybr Green I, 0.1 µM of primer and 10 nM fluorescein (Life Technologies, Grand Island, NY, USA) Relative target Ct (the threshold cycle) values of Bcl-2 and Bax were normalized to GAPDH, as housekeeping gene. The mRNA levels of cells treated with ethanolic and water ASE extract were expressed using the $2^{-\Delta\Delta Ct}$ method (Kenneth and Schmittgen, 2001), relative to the mRNA level of the untreated sample for each experiment.

II.2.8 Statistical analysis

Results are expressed as mean ± standard deviation (SD) and were analyzed by ANOVA with Duncan's multiple range tests procedure (DMRT) and Student's t-test using 25.0 SPSS Windows software. Differences were considered significant for $p < 0.05$.

Chapter III :
Results and Discussion

III.1 The Effects of grape Leaves Extract on hyperhomocysteinemia induced inflammatory endothelial damage in cardiovascular diseases

As shown in figure 14, the total Hcy levels in groups F (7.56 ± 0.21 µmol/l), M (12.20 ± 0.78 µmol/l), MP (9.06 ± 0.34 µmol/l) and P (7.24 ± 0.40 µmol/l) were showed a significant difference between groups in mice during 15 days of treatment $P \leq 0.05$ (Figure 14). The Tukey test was showed that the homocysteine concentration in mice administered with L-methionine was increased highly significantly in group M when it was compared to the control group $P \leq 0.05$. However the homocysteine concentration was decreased significantly in the group of mice administered with L-methionine and treated with *Vitis vinifera* L when it was compared to the group (M) $P \leq 0.05$. Exceptionally, no significant changes were observed between the group treated only by *Vitis vinifera* leaves and the control group ($P > 0.05$) (Figure 14).

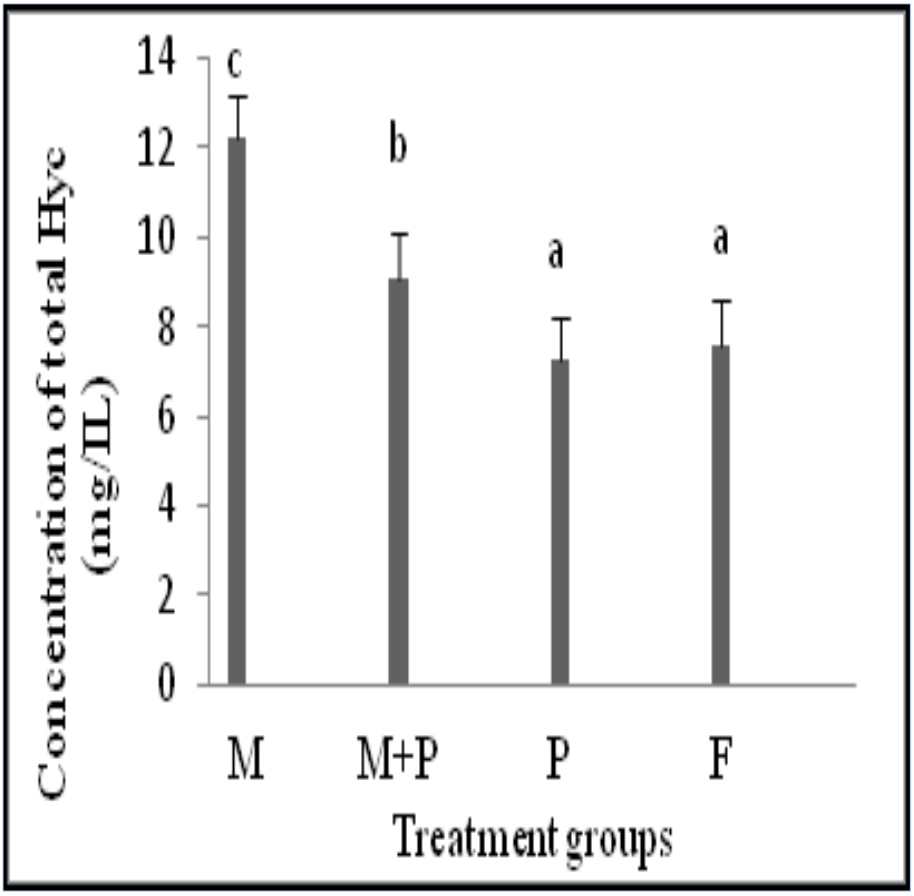

Figure 14: Total Hcy level in the groups treated during 15 days. Uncommons letters assigned nonsignificant means.

Homocysteine (Hyc) is considered as a risk marker and can be used for screening patients of high menace for cardiovascular events (Refsum et al., 1998). In this study, the oral administration of high dose of L-methionine 1 g/kg/day during 15 days showed a significant increase in the level of plasma tHyc compared to the control group. These results are in agreement with those found by (Zerizer, 2006), who showed that the high oral methionine load is the direct cause of the elevation of the total homocysteine (tHcy), the sum of all homocysteine forms that exist in plasma or serum, therefore having hyperhomocysteinemia means the elevation of total homocysteine (Bernardo et al., 2004). Two hypotheses were formulated to explain the atherogenicity of hyperhomocysteinemia. The lipid hypothesis stipulates that the alteration of lipoprotein metabolism secondarily induces an involvement of the vascular wall, and the inflammatory hypothesis is dominated by the direct aggression of the cells and vascular connective tissue (Demuth et al., 2000).

The concentrations of hs-CRP in groups F (0.14 ± 0.012 mg/l), M (0, 28 ± 0,019 mg/l), MP (0.18 ±0.016 mg/l) and P (0.14 ± 0,012) were showed a significant difference between groups $P \leq 0.05$ (Figure 15). The Tukey test was revealed that the hs-CRP concentration in the group (M) was increased highly significantly when it was compared to the groups (F) and (P) $P \leq 0.01$. However the concentration of hs-CRP was decreased significantly in the group (MP) when it is compared to the group (M).

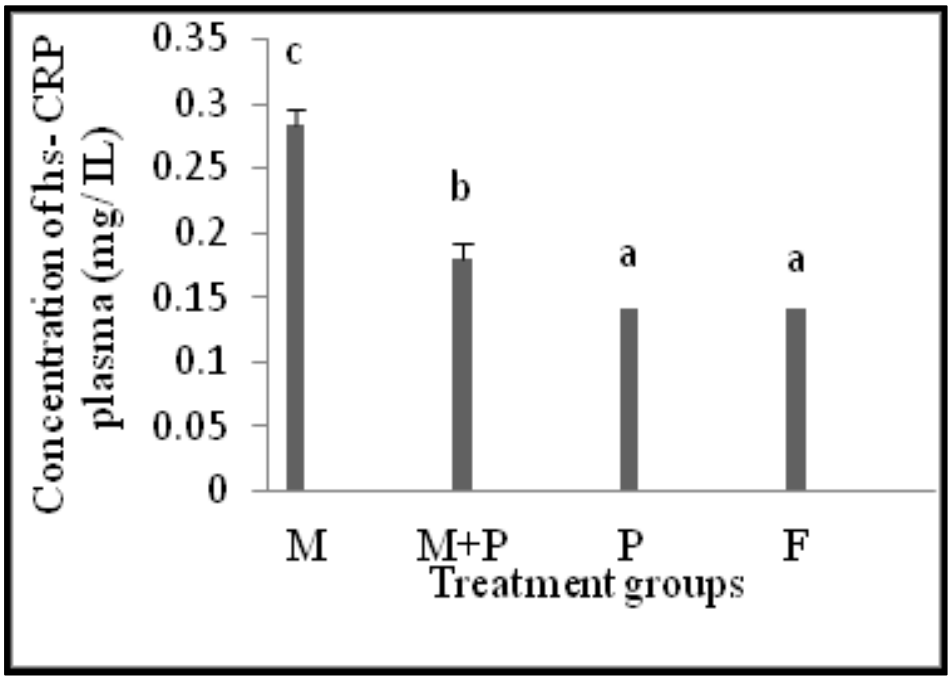

Figure 15: Concentration of hs-CRP plasma in the groups treated during 15 days. Uncommons letters assigned nonsignificant means.

Currently and in several scientific researches, the CRP is used as a marker of cardiovascular risk (Folsom et al., 2002). The group (M) showed significant high level of hs-CRP compared with the control one. Benmebarek et al. (2013) confirmed that methionine at dose of 200 mg/kg/day administered to mice, during the 21 days period, increased significantly the levels of plasma hs-CRP. This result was considered as an initiative of the inflammatory process, which was confirmed by the histological investigation of the aorta. Results are in agreement with the previous experimental studies of Benmebarek et al. (2013) who found that Hyperhomocysteinemia as angiotoxic and toxic activity explained by the loss and degeneration of the endothelium, formation of foam cells in the different sections of the aorta, change in the smooth muscle cells nuclei forms from a fusiform aspect to a rounded appearance, and the alteration of the cardiac muscle and liver necrosis. In addition, Zerizer and Naimi (2004) reported the structural alterations in the aorta, heart and liver caused by the administration of high doses of methionine.

Results demonstrated that the concentration of the glutathione reduced in groups F (29.28 ± 1.48 nmol/mg), M (19.38 ± 1.21 nmol/mg), MP (26.74 ± 1.06 nmol/mg) and group P (30.17 ± 1.37 were showed a significant difference between groups $P \leq 0.05$ (Figure 16).

The Tukey test showed that the concentration of the glutathione reduced in group M was decreased highly significantly when it is compared to the control group $P \leq 0.01$. However the concentration of GSH was increased in the groups treated with *Vitis vinifera* L.

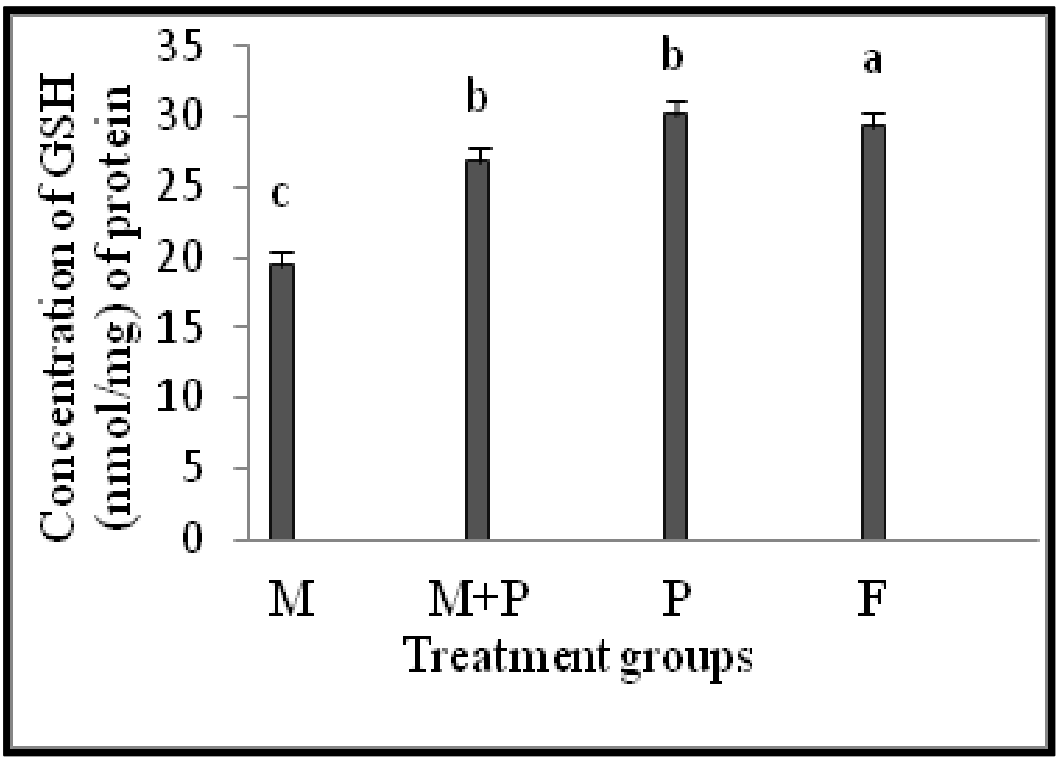

Figure 16: Concentration of GSH in the groups treated during 15 days. Uncommons letters assigned nonsignificant means.

Substantially reduced (GSH) involved in maintaining the redox potential of the cell cytoplasm and in a number of detoxification reactions and scavenging reactive oxygen species (Haleng et al., 2007). Result demonstrated that the group (M) showed a significant decrease in the level of GSH compared to the control group (F). These results confirmed an oxidative stress generated by the reactive sulfhydryl group (-SH) in the homocysteine (Jacobsen, 2000), which is quickly oxidized, leading to the formation of Hcy, mixed disulfides and Homocysteinethiolactone. The oxidation of the -SH group generates superoxide anion O_2•, hydrogen peroxide H_2O_2 and hydroxyl radicals OH- (Zitoun, 1998).

Zeng et al. (2004) confirmed that the Hcy induced the production of MCP1 and IL-8. Additionally, a recent study suggested that Hcy induced the production of O_2• in vascular smooth muscle cells (Wang and OK., 2001[a]). The group (MP) treated by leaf of *Vitis vinifera* and L-methionine (500 mg/kg and 1 g/kg) noted a significant decrease in the levels of tHyc compared to group (M). The same group rectified significantly the level of hs CRP compared with the control group and group (M). At the same time, the group (MP) re-established significantly the level of GSH compared to the group (M).

Aorta histological

The results of the histological investigation showed a clear modification in the aorta. The group (M) which was treated with 1 g/kg of L-methionine appeared with oval nuclei of muscular fiber, desquamation of endothelial cells and muscular lysis (Figure 18, 21, 22, 23 and 26). However, in the control group (F), the aortic sections were showed an intact endothelium (Figure 17, 20 and 25). The group (MP) which was treated with leavesextracts of the *Vitis vinifera* and 1 g/kg of L-methionine was showed only slight modifications including some oval nuclei of muscular cells figure (19, 24 and 27).

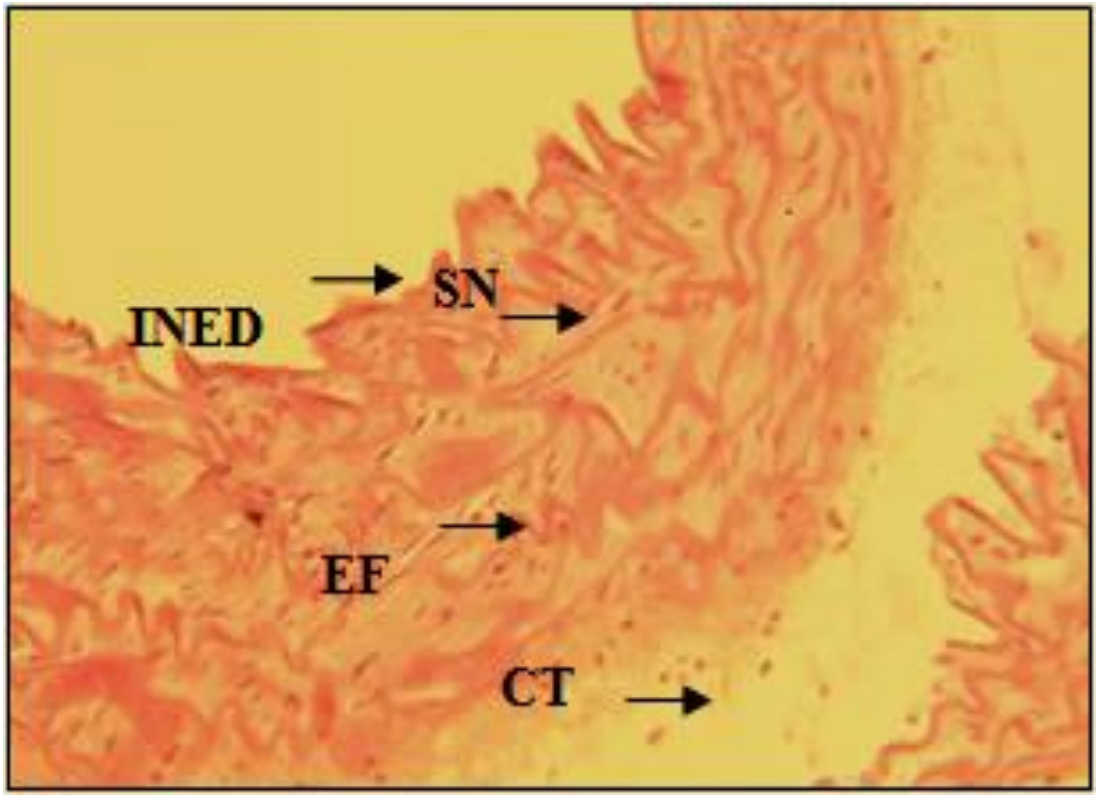

Figure 17: Longitudinal section of arch aorta 15 days of flour.Heamatoxylin Eosin staining (x100). EF: elastic fiber, SN: spindle nuclei, CT: connective tissue, INED: intact endothelium

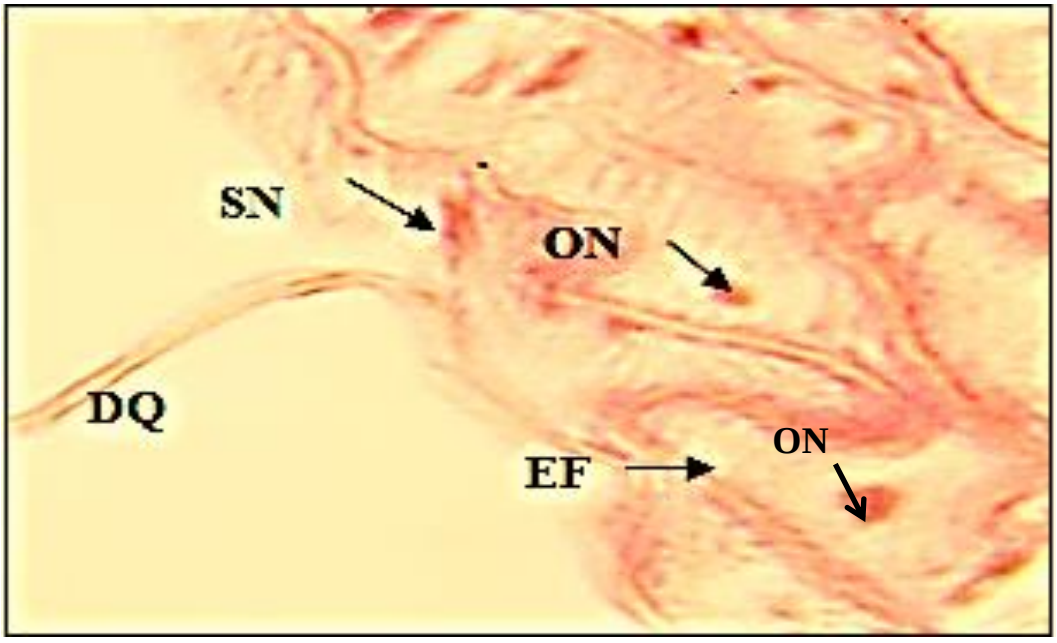

Figure 18: Longitudinal section of arch aorta 15 days of L-methionine. Heamatoxylin Eosin staining (x400). EF: elastic fiber, DQ: desquamation, ON: oval nuclei, SN: spindle nuclei

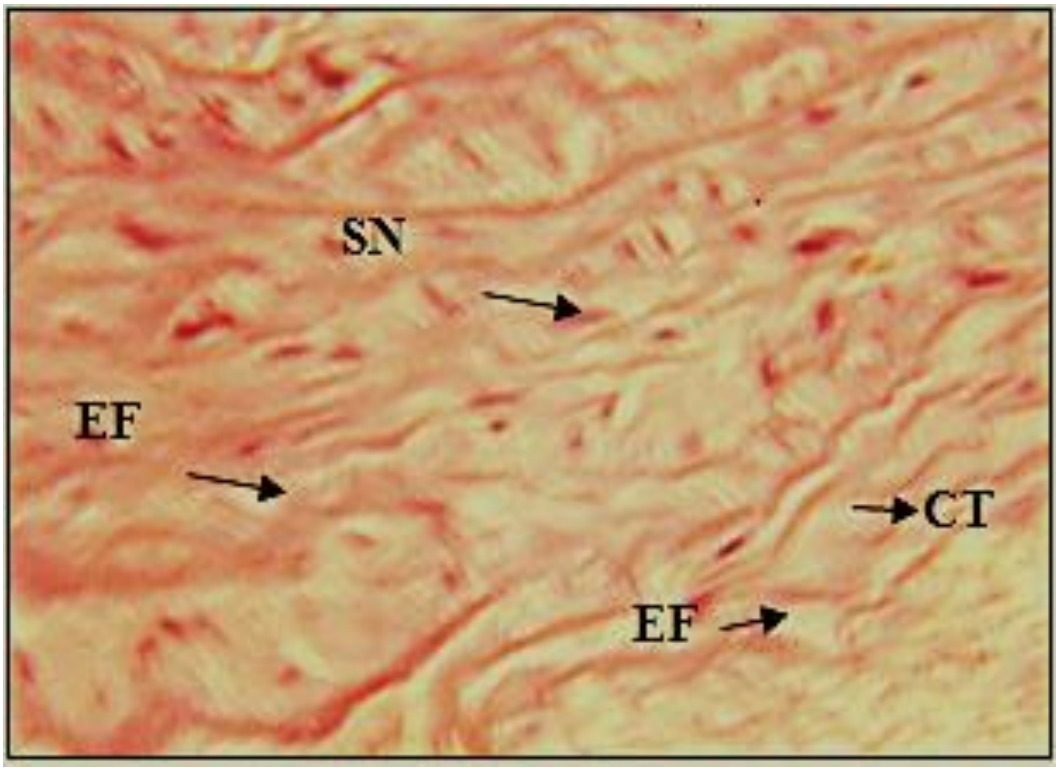

Figure 19: Longitudinal section of arch aorta 15 days of L-methionine and *Vitis vinifera* leaves. Heamatoxylin Eosin staining (x100). EF: Elastic Fiber SN: Spindle nuclei CT: connective tissue

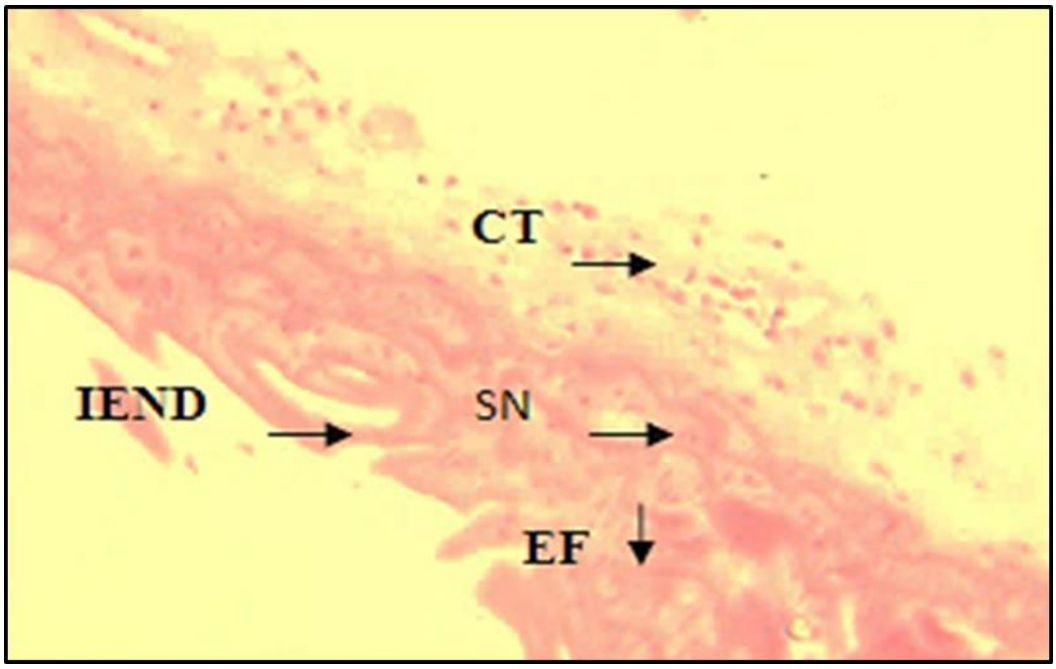

Figure 20: Longitudinal section of abdominal aorta 15 days of flour. Heamatoxylin Eosin staining (x100) EF: Elastic Fiber SN: Spindle nuclei CT: connective tissue IEND: Intact Endothelium

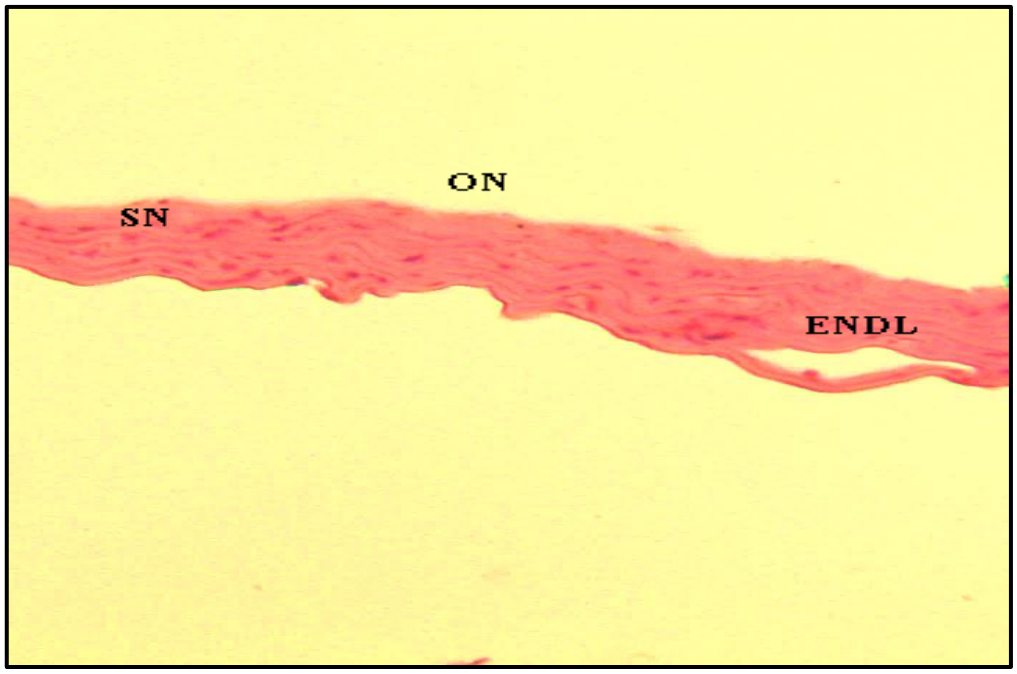

Figure 21: Longitudinal section of abdominal aorta 15 days of L-methionine. Heamatoxylin Eosin staining (x100). ENDL: Endolysis ON: Oval nuclei SN: Spindle Nuclei

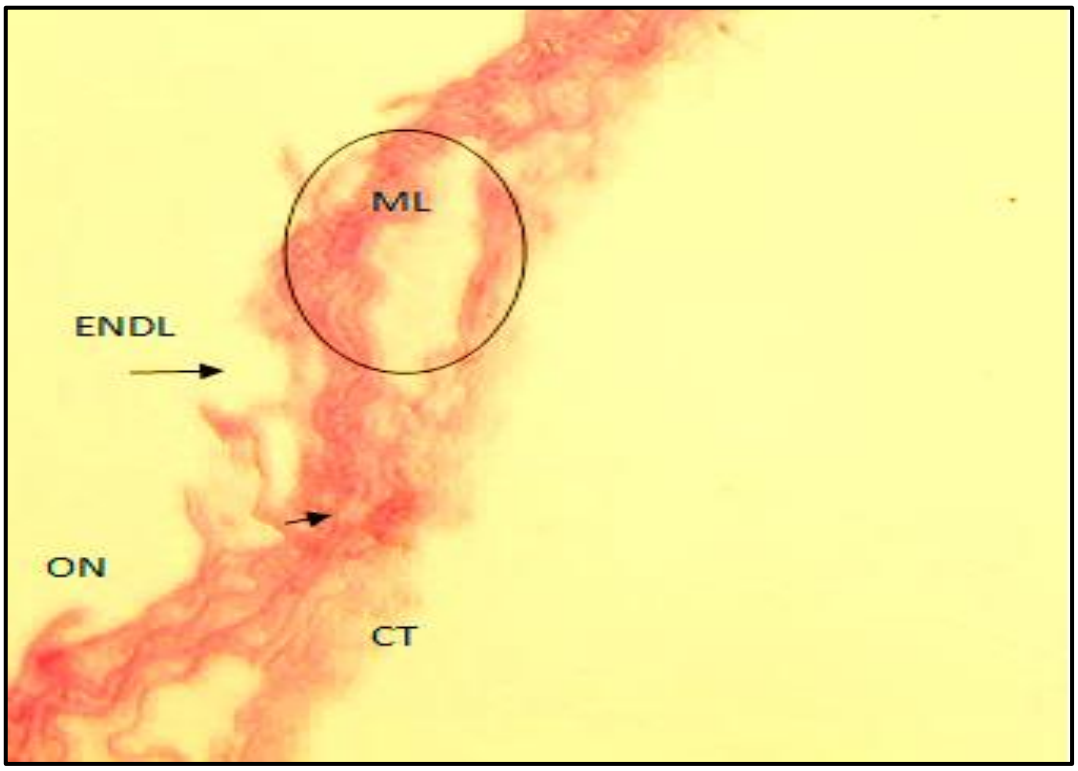

Figure 22: Longitudinal section of thoracic aorta 15 days of L-methionine. Heamatoxylin Eosin staining (x100). ENDL: Endolysis ML: Muscular Lysis CT: connective tissue.

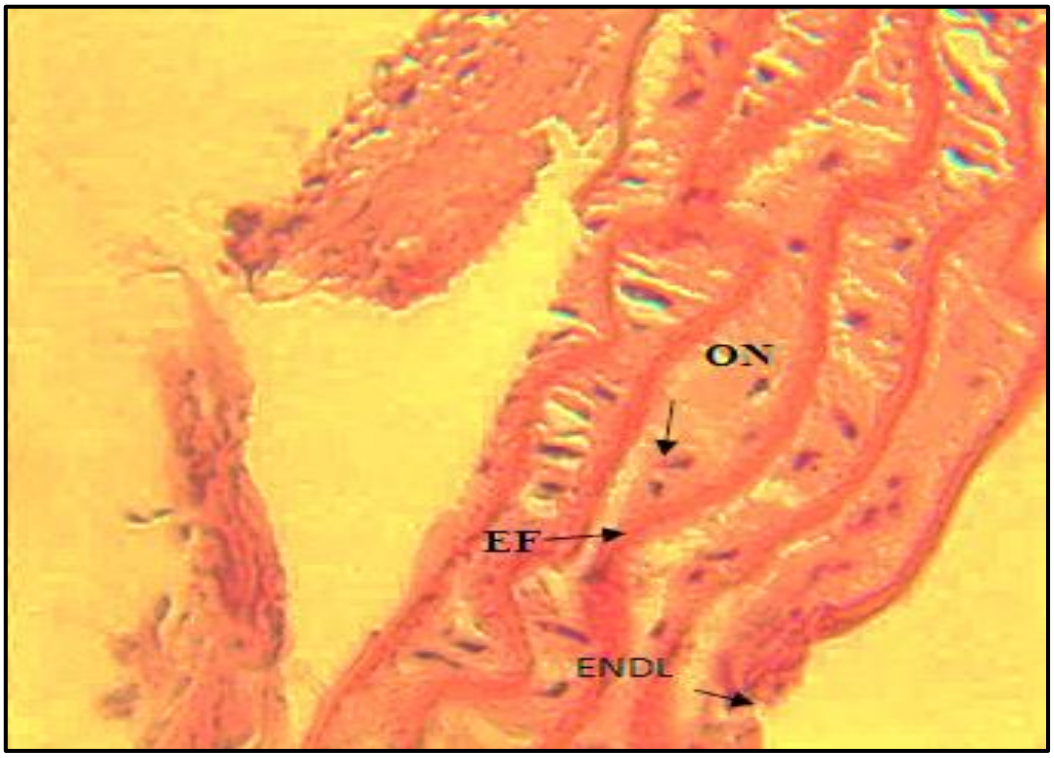

Figure 23: Longitudinal section of thoracic aorta 15 days of L-methionine.Heamatoxylin Eosin staining (x400). EF: Elastic Fiber ON: Oval nuclei ENDL: Endolysis

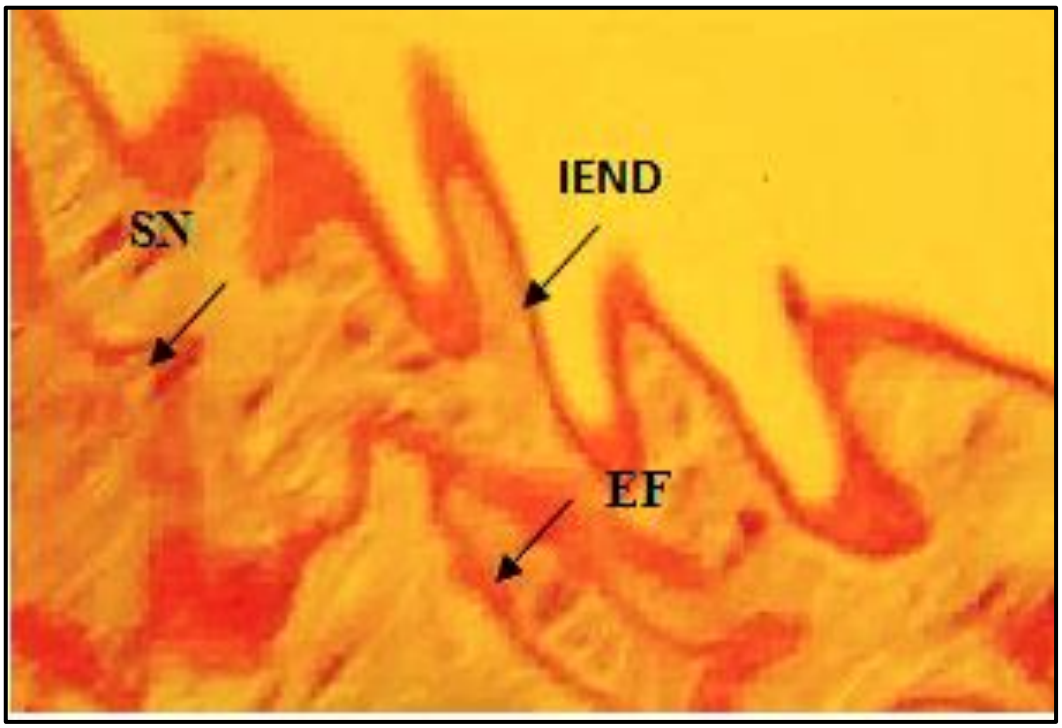

Figure 24: Longitudinal section of thoracic aorta 15 days of L-methionine and *Vitis vinifera*. Heamatoxylin Eosin staining (x400). EF: Elastic Fiber SN: Spindle Nuclei INED: Intact Endothelium

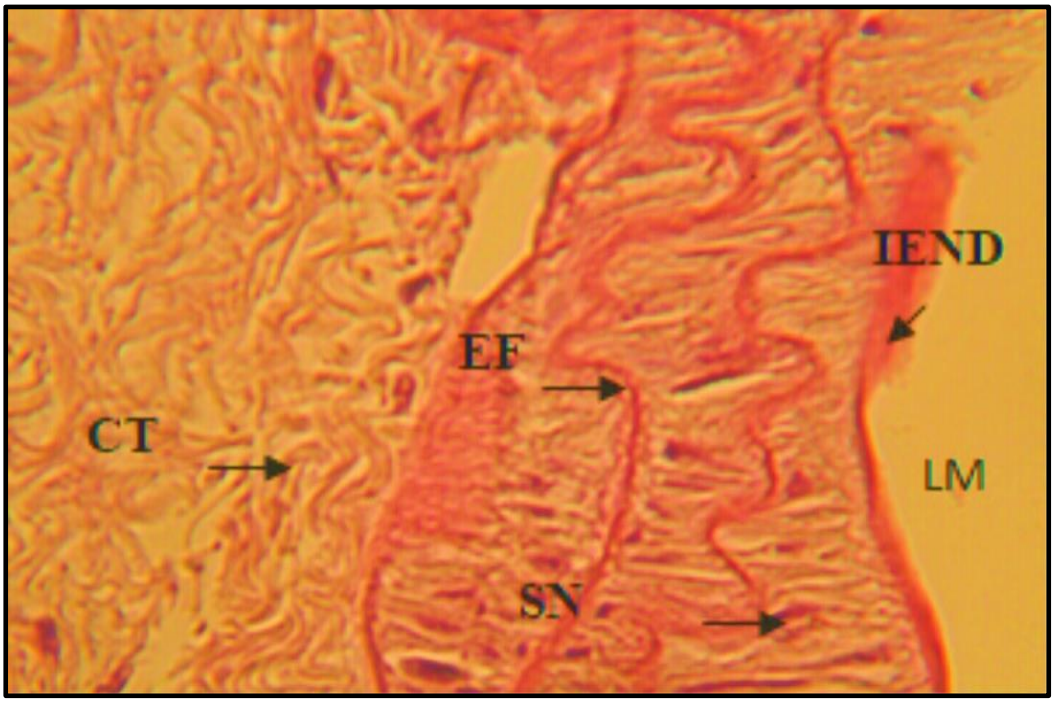

Figure 25: Longitudinal section of iliac aorta 15 days of flour. Heamatoxylin Eosin staining (x100) EF: Elastic Fiber SN: Spindle nuclei CT: connective tissue IEND: Intact Endothelium LM: Lumen

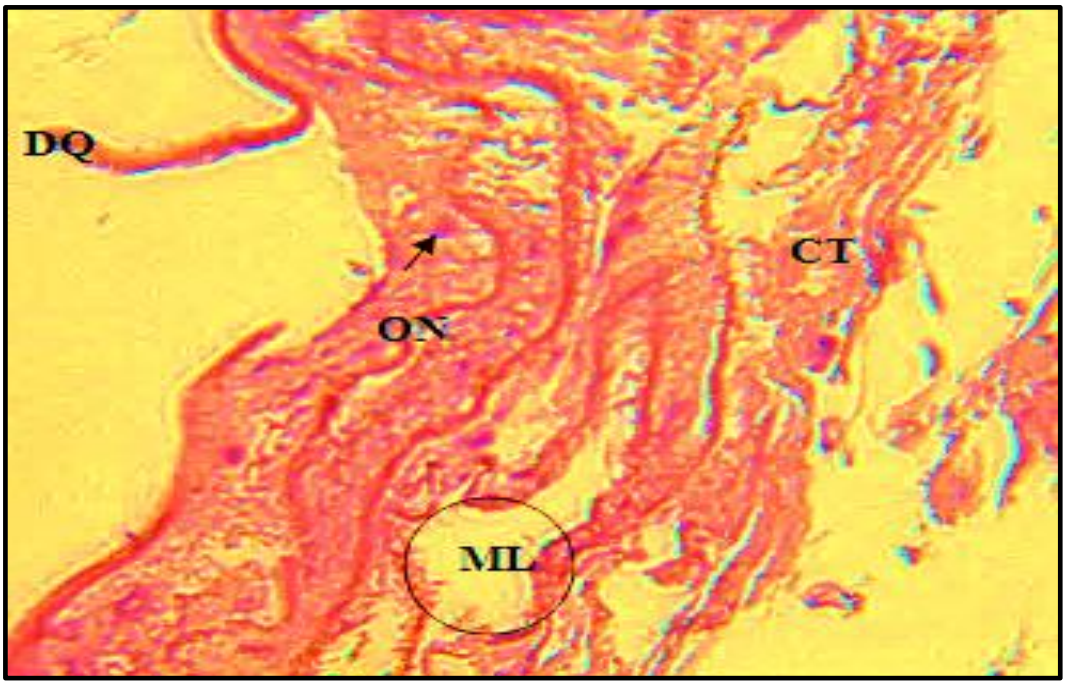

Figure 26: Longitudinal section of iliac aorta 15 days of L-methionine. Heamatoxylin Eosin staining (x100). EF: Elastic Fiber DQ: Desquamation ON: Oval nuclei Nuclei ML: Muscular Lysis

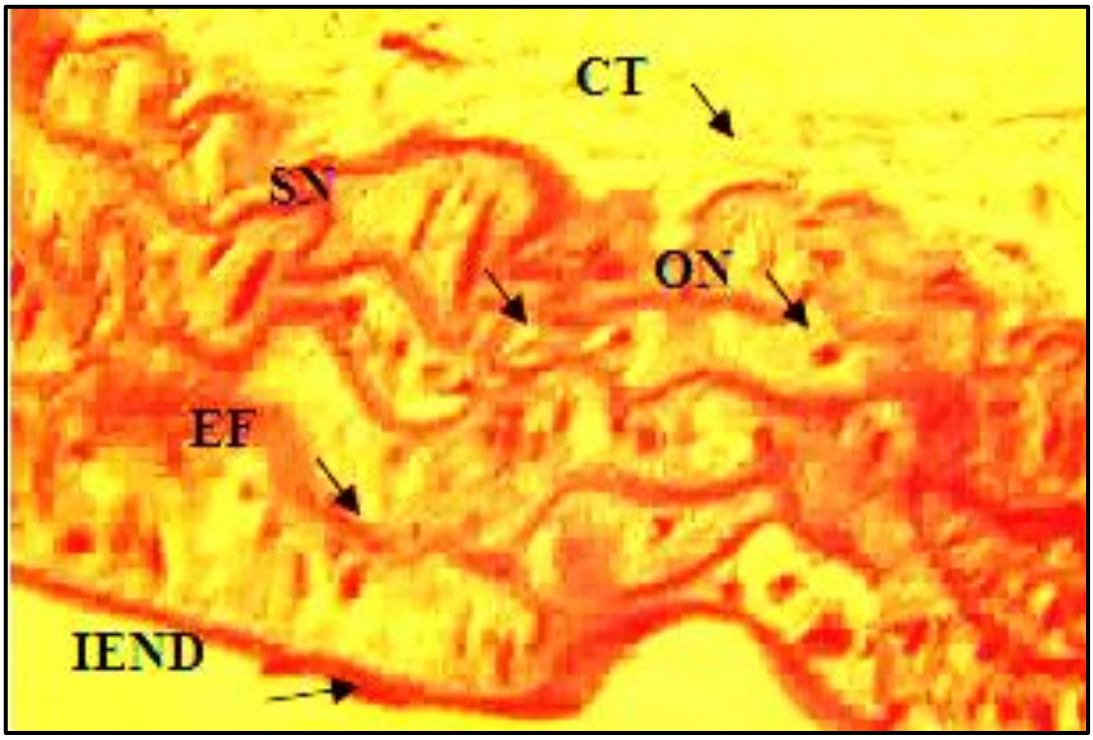

Figure 27: Longitudinal section of iliac aorta 15 days of L-methionine and *Vitis vinifera.* Heamatoxylin Eosin staining (x100). EF: Elastic Fiber SN: Spindle nuclei IEND: Intact Endothelium ON: Oval Nuclei CT: connective tissue

These significant relationships between the parameters can be explained as the effect of the existed phenols in the GLAV. Exactly, the group (MP) treated by the GLAV was able to restore the level of the hs-CRP and GSH and maintain the correlation between the three parameters and could correct the damaged cells in aorta.

The results confirmed that GLAV (500 mg/kg/day) has an antioxidant and anti-inflammatory effects induced by Hyperhomocysteinemia in mice treated by a high dose of L-methionine 1 g/1 kg/day during 15 days. Benmebarek et al. (2013) asserted that *S. mialhesi* extract lowered the plasma hs-CRP and corrected the damaged cells. Yalçinkaya et al. (2009) demonstrated that a high methionine diet induced oxidative stress in serum, heart, and aorta in rabbits. Also, recent study conducted on humans' patients indicating that elevated Hcy levels are correlated with cardiovascular aortic vascular diseses patients compared with healthy controls (Guandi et al., 2018).

III.2 Phenolic composition of extract GLAV (*Vitis vinifera*)

The HPLC/DAD/ESI-MS analysis was used to identify all compounds directly, or by comparing the results to literature, and the analysis of leaf ingredients were permitted the identification of cyanidin-3-glucosides, -3-(6-acetyl) glucosides, and -3-(6-p-coumaroyl)glucosides (Table 4; Figure 28). Nonanthocyanin phenolic compounds identified in leaf ingredients included the flavonols quercetin-3-O-galactoside, quercetin-3-O-glucuronide, quercetin-3-O-glucoside, kaempherol-3-O-galactoside, kaempherol- 3-O-glucuronide, kaempherol-3-O-glucoside ansd quercetin. The Trans-caftaric-acid is the only cinnamates identified in our extract (Table 5; Figure 29).

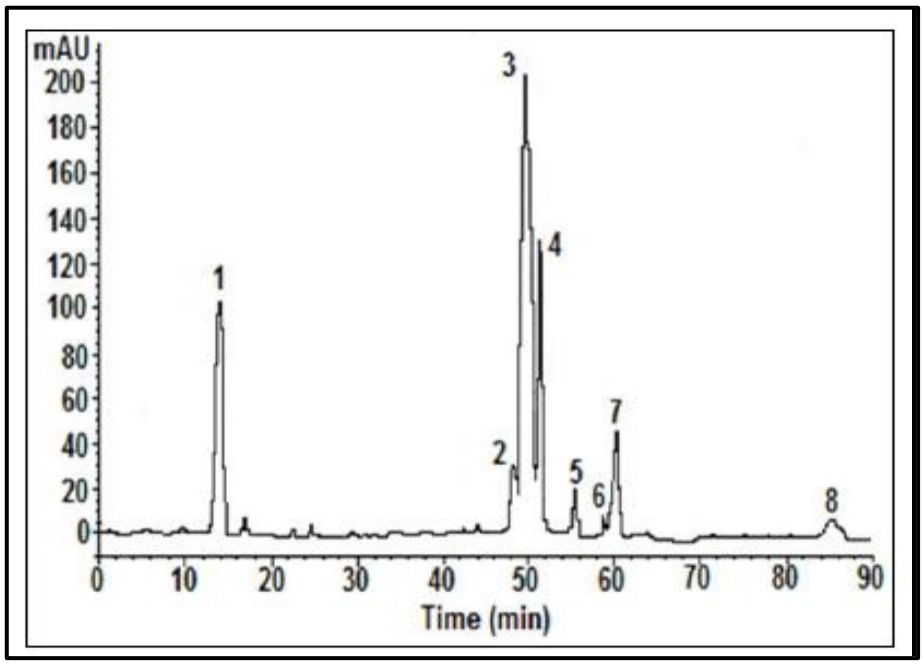

Figure 28: HPLC-DAD Chromatogram at 340 nm of non-anthocyanin compounds from a *Vitis vinifera* L. leaves extract

(1) trans-caftaric acid; (2) Quercetin-3-O-galactoside; (3) quercetin 3-0-glucuronide; (4) quercetin 3-0-glucoside; (5) kaempferol 3-0-galactoside; (6) kaempferol 3-0-glucuronide; (7) kaempferol 3-0-glucoside; (8) quercetin.

Table 4: Characteristics of anthocyanins (a-n) and flavonols (2-8) detected in a methanol extract of *Vitis vinifera* L. leaves, according to the retention time (tR), mass spectral details, UV data (λmax), corroborated by references.

Peaks	$^{x}t_R$ (min)	Compound	$[M+H]^+$ (m/z)	$[M-H]^-$ (m/z)	λmax	Fragments (MS/MS)	Reference
A	9.8	Delphinidin 3-glucoside	465.2	-	522	303	Villiers et al. (2004); Kammerer et al. (2004)
B	11.1	Cyanidin 3-glucoside	449.3	-	514	287	
C	12.6	Petunidin 3-glucoside	479.4	-	522	317	
D	13.8	Peonidin 3-glucoside	463.4	-	515	301	
E	15.0	Malvidin 3-glucoside	493.1	-	524	331	
F	18.8	Cyanidin 3-(6-p-acetyl) glucoside	495.4	-	519	287	Villiers et al. (2004)
G	22.3	Peonidin 3-(6-acetyl) glucoside	505.0	-	516	301	
H	23.2	Delphinidin 3-(6-p-coumaroyl) glucoside	611.3	-	527	303	Villiers et al. (2004); Kammerer et al. (2004)
I	25.3	Cyanidin 3-(6-p-coumaroyl) glucoside	595.4	-	522	287	
L	26.3	Petunidin 3-(6-p-coumaroyl) glucoside	625.2	-	536	317	
M	28.8	Peonidin 3-(6-p-coumaroyl) glucoside	609.2	-	520	301	
N	30.5	Malvidin 3-(6-p-coumaroyl) glucoside	639.0	-	517	331	
1	13.9	Trans-caftaric acid	-	311.0	320	179	Kammerer et al. (2004)
2	49.2	Quercetin 3-0-galactoside	-	463.1	256	301	Kammerer et al. (2004); Flamini et al. (2015)
3	50.1	Quercetin 3-0-glucuronide	-	479.1	256	301	
4	51.7	Quercetin 3-0-glucoside	-	463.1	256	301	

5	55.6	Kaempferol 3-0-galactoside	-	447.3	262	285	Villiers et al. (2004); Flamini et al. (2015)
6	59.0	Kaempferol 3-0-glucuronide	-	463.1	262	287	Flamini et al. (2015
7	61.5	Kaempferol 3-0-glucoside	-	477.0	262	285	Kammerer et al. (2004); Flamini et al. (2015)
8	85.5	Quercetin	-	304.0	254	273	Kammerer et al. (2004)

$^{x}t_R$Mean retention time of 3 runs

Among the extracted polyphenols from the Algerian *Vitis vinifera* L. leaves, the anthocyanins are the main chemical group, about 80.34 µg in each 1 g of freeze-driedsample (Table 5, Figure 29). We have 12 compounds identified by HPLC-DAD as follows: the peonidins, as the main chemical group, with about half (46.50%) of the total anthocynins with 37.35 µg/g freeze-dried sample in each g of the predominant compound was peonidin 3-glucoside, the second abundant group was the cyanidins, with cyanidin-3-glucoside of 78% per the total of cyanidins tailed by cyanidin-3-(6-acetyl)-glucoside (13%) and cyanidin-3-(6-*p*-coumaroyl)-glucoside (9%). The malvidins represented the third abundant group with 12.98 µg/g of sample and 2 compounds (malvidin 3-glucoside and malvidin 3-(6-*p*-coumaroyl) glucoside), the glucoside forms were the most abundant; the two remaining chemical classes, delphinidins and petunidins were contributed to total anthocyanins for 6.30% and 2.63%, respectively. Concerning the non-anthocyanins it is interesting to note the high concentrations of trans-caftaric acid and quercetin 3-*0*-glucuronide. Quercetins were the most abundant flavonols and were represented by quercetin (5.2%) and the glucuronide (76.5%), galactoside (8.3%) and glucoside (10%) forms. Kaempferols were presented 11.40% of flavons and the most abundant form was the galatoside (82.2%) one. For these compounds it is interesting to note the very high concentration of the glucuronide form and the notable concentration of quercetin.

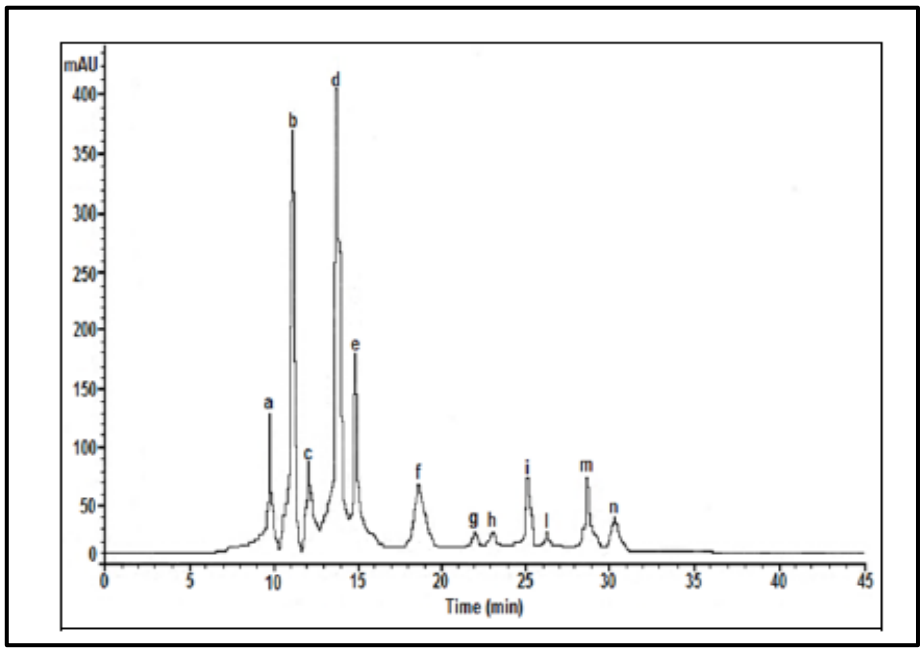

Figure 29: HPLC-DAD Chromatogram at 530 nm of anthocyanin compounds from a *Vitis vinifera* L. leaves extract

(a) delphinidin 3-glucoside; (b) cyanidin 3-glucoside; (c) petunidin 3-glucoside; (d) peonidin 3-glucoside; (e) malvidin 3-glucoside; (f) cyanidin 3-(6-p-acetyl) glucoside; (g) peonidin 3-(6-acetyl) glucoside; (h) delphinidin 3-(6-p-coumaroyl) glucoside; (i) cyanidin 3-(6-p-coumaroyl) glucoside; (i) petunidin 3-(6-p-coumaroyl) glucoside; (l) petunidin 3-(6-p-coumaroyl) glucoside; (m) peonidin 3-(6-p-coumaroyl) glucoside; (n) malvidin 3-(6-p-coumaroyl) glucoside.

Table 5: Phenolic composition of *Vitis vinifera* L. leaves extract according to HPLC-DAD chromatography

Anthocyanins[x]					
Cyanidins	**Delphinidins**	**Petunidins**	**Peonidins**	**Malvidins**	**Total anthocyanins**
22.83 µg	5.06 µg	2.12 µg	37.35 µg	12.98 µg	80.34 µg
28.42%[y]	6.30%	2.63%	46.50%	16.15%	
Flavonols[z]					
Quercetins			**Kaempherols**		**Total flavanols**
39.70 µg			5.11 µg		44.81 µg
88.60%[w]			11.40%		
Cinnamates					
Trans-caftaric acid					
9.52 µg					

[x]Expressed as µg malvidin-3-glucoside equivalents/g freeze-dried leaves

[y]% of total anthocyanins: Σ Cyandins = cyanidin-3-glucoside + cyanidin-3-(6-acetyl)-glucoside + cyanidin-3-(6-p-coumaroyl)-glucoside; Σdelphinidins = delphinidin-3-glucoside + delphinidin-3-(6-p-coumaroyl)-glucoside; Σ petunidins = petunidin-3-glucoside + petunidin 3-(6- p-coumaroyl)-glucoside; Σ peonidins = peonidin-3-(6-acetyl)-glucoside + peonidin-3-(6-p-coumaroyl)-glucoside; Σ malvidins = malvidin-3-glucoside + malvidin-3-(6-p-coumaroyl)-glucoside

[z]Expressed ad µg quercetin or keampherol equivalents/g freeze-dried leaves

[w]% of total flavonols: Σ Quercetins = quercetin-3-O-galactoside + quercetin-3-O-glucuronide + quercetin-3-O-glucoside + quercetin; Σ kaempherols = kaempherol-3-O-galactoside + kaempherol-3-O-glucuronide + kaempherol-3-O-glucoside

The anthocyanin/flavonol ratio = 1.79

Many studies demonstrate that grapes are rich in anthocyanins, flavanols, flavonoids, terpenes, organic acids, vitamins, carbohydrates, lipids and enzymes (Mazza et al., 1993 and Felicio et al., 2001). These findings have created considerable interest in grape leaves as a promising source of compounds with nutritional properties and biological potential. Moreover, the use of grape leaves provides a way of solving the disposal problems arising from the large amounts of industrial residues generated by the wine and juice industries (Monagas et al., 2006 and Wang and Weller, 2006[b]).

In this study, the High-performance liquid chromatography (HPLC) was used for the separation and quantification of polyphenols in leaves of *Vitis vinifera*. L species, the Algerian variety. The results showed that high levels of phenols; anthocyanins, flavonols and trans-caftaric acid. The results is agrees with the work of (Monagas et al., 2006) who reported that HPLC-DAD/ESI-MS analysis of V. Vinifera spp. leaves ingredient allowed the identification of anthocyanidin-3-glucosides,-3-(6-acetyl)glucosides, and -3-(6-p-coumaroyl) glucosides and the flavonols quercetin-3-O-glucoronide, quercetin-3-O-glucuronide, quercetin-3-O-glucoside, kaempherol-3-O-galactoside, kaempherol-3-O-glucuronide, kaempherol-3-O-glucoside, and quercetin like non-anthocyanin content. The chromatograph pattern and the compounds identified agree with other paper on the same subject (Monagas et al., 2006). Trans-Caffeoyltartaric acid (trans-caftaric acid) was the only hydroxycinnamic acid derivative identified in the studied leaf ingredients and results obtained were in agreement also with (Monagas et al., 2006). According of the results

obtained, GLAV contain much higher concentration of peonidins, Trans-Caffeoyltartaric acid (trans-caftaric acid) and Quercetins but the concentration of Kaempherols is found inferior than values mentioned by the results of Monagas et al. (2006). The differences observed between the results may be attributed to the period of the plant growth cycle, variety, cultivar conditions, weather and finally to the processing and preparation because leaves used by Monagas et al. (2006) were cultivated using the commercial dietary technology.

III.3 Yield and total phenolic contents

In this part, we investigated the accelerator solvent extraction method to prepare crude extracts of grape leaves, grown in Algeria, in ultrapure water and 60% ethanol. We aimed at evaluating the anti-proliferative effects of these extracts on HepG2 hepatocarcinoma cells and MCF-7 breast cancer cells. The amount of total phenols and the antioxidant activity were evaluated by scavenging DPPH• and trapping of hydroxyl radical using EPR-spin trapping technique. Then, cell viability was analyzed by using different concentrations of the extracts.

Table 6 shows the yield and total phenolic content of ethanolic and water ASE crude extracts obtained from grape leaves. The water extract gave a higher total phenolic yield (22.8 ± 3.21%) as compared to ethanol (18.87 ± 0.6 %), despite not being statistically significant (p = 0.116). However, the ethanolic extracts exhibited larger amount of TP (around 2.8 times) as compared to water extract (p=0.001). The ethanol polarity might be responsible for the observed TP content difference.

Table 6: Yield extraction (%), total phenols, ERP-spin trapping and DPPH-radical scavenging activity (IC50) of ethanolic and water ASE crude extracts of grape leaves.

	TP (mg GAE/gr DW±SD)	**Yield (%±SD)**	**IC50 ERP (mg/ml ± SD)**	**IC50 DPPH (mg/ml ±SD)**
WACE	55,41±0,11[a]	22,8±3,21[a]	0,67±0.53[a] R^2 = 0,9791	0,15 ± 0.41[a] R^2 = 0,9711
EACE	155,73±1,20[b]	18,87±0,6[a]	0.64±0.71[a] R^2 = 0,9989	0,09 ± 0,32[b] R^2 = 0,9922

DW: Dry weight, SD: All results are presented as mean ± standard deviation, WACE: Water ASE crude extract, EACE: Ethanolic: ASE crude extract, IC50: the

concentration of a sample at which 50% inhibition of free radical activity is observed; GAE: Gallic acid equivalent, common letter are not significantly different.

Results showed for the first time, the extraction of bioactive compounds such as phenolic compounds from grape leaves by ASE. ASE provided fast (10 min), easy (automated technique), safe (no direct exposure to the solvent) and inexpensive (in 34 ml of solvent) extraction, leading to high yields and high phenolic contents. Leelavinothan and Arumugam (2008) found that grape leaves contain 99 mg of gallic acid equivalents (mg GAE)/g of phenolic compounds in 70% hydroalcoholic solvent after 72 h of extraction (Pari and Suresh, 2008), a value lower than the one obtained with the extraction methods described in the present study and requiring a longer extraction time and more solvent. Orhan et al., (2007) describe a phenolic compound yield of 16,07% by extracting 500 g of *Vitis vinifera* dried powder leaves with 80% ethanol at room temperature (5 L * 6 times) (Deliormanet al .,2007). The pressure exerted by ASE allows the extraction cell to be filled faster and helps to force liquid into the solid matrix. Elevated temperatures enhance the diffusivity of the solvent, resulting in an increased extraction kinetic (Richter et al., 1996, Brachet et al., 2001 and Kaufmann et al., 2001). Consequently, ASE may be used to obtain a higher yield in an extremely short time as compared to all previously described methods. Indeed, in recent years, ASE has been successfully applied to the extraction of phenolic compounds from different plant materials, such as grape seeds and skin (Ju and Howard, 2003, Pineiro et al., 2006 and Luque-Rodriguez et al., 2007) apples (Alonso-Salces et al., 2001), spinach (Howard and Pandjaitan, 2008), eggplants (Luthria and Mukhopadhyay, 2006) and barley flours (Bonoli et al., 2004).

III.4 DPPH and ERP radical-scavenging activity

The antioxidant capability was expressed as the quantity of antioxidant inducing a 50% decrease of DPPH concentration or a 50% inhibition of the hydroxyl radical production (IC50) (Table 6). The trapping efficiency of DPPH or hydroxyl radical is inversely proportional to (IC 50). Table 6 showed the (IC50) of grape leaves ethanolic and water crude extracts. The ethanolic extract of grape leaves showed a higher activity of scavenging DPPH radical (0.09 mg/ml) as compared to the water extract (0.15 mg/ml)($p= 0.035$). Ethanolic and water extracts provided respectively IC50 0.67(±0.53) and 0.64 (±0.71) mg/ml. The trapping of hydroxyl radical did not show any significant difference between the two extracts ($p=0.181$).

Oxidative stress is a pathogenetic mechanism associated with several diseases, including atherosclerosis, neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, cancer, diabetes mellitus, inflammatory diseases, as well as psychological diseases or aging processes (Dürackova, 2010). Indeed, increased formation of free radicals (FR) can promote the development of malignancy, and "normal" rates of FR generation may account for the increased risk of cancer development in the elderly (Barry, 2007).

Electron paramagnetic resonance (EPR) spin trapping has become an indispensable tool for the specific detection of reactive oxygen free radicals in biological systems (Hawkins, and Davies, 2014). The EPR spin-trapping technique was used to study the ability of ASE grape leaves extracts to quench OH radicals, which are common reactive oxygen species associated with oxidative cell damage (Goupy et al., 2003). The hydroxyl radical reacts unselectively and very quickly with any chemical compound able to lose a hydrogen atom (Fadda et al., 2018). Our results indicate that water and ethanol grape leaf extracts possessed similar ·OH radicals quenching activity. In water extract, the content of TP, despite being lower than that of ethanol, was high enough to react with the hydroxyl radicals produced, thus excluding any dose-dependent mechanism in the reaction between antioxidants and ·OH.

DPPH• free radical was used to evaluate the ability of phenolic compounds to transfer labile hydrogen atoms to radicals (Hawkins and Davies, 2014). Our extracts showed high capability to scavenge DPPH•, due to the presence of different polyphenols, including flavonoids, which can be found in grape leaves (Farhadi et al., 2016 and Samoticha et al., 2017). Generally, the chemical structure of flavan-3-ol family grants a good antioxidant response towards DPPH•. The hydrogen-donating substituents (hydroxyl groups), attached to the aromatic ring structures of flavonoids, allow for a redox reaction able to scavenge free radicals (Brand-Williams and Cuvelier, 1995 and Deliorman et al., 2007).

III.5 Effect of grape leaves Ethanolic and Water extract on HUVEC Cell Proliferation

Table 7: IC50 of grape leaves ethanolic and water ASE crude extracts (EACE and WACE) respectively on MCF-7, HepG2 and HUVEC cells

Cells / Extract	MCF-7	HepG2	HUVEC
WACE IC50 (mg/ml)	0.71	1.1	>> 2
EACE IC50 (mg/ml)	0.43	0.7	>> 2

IC50: the concentration of a sample at which 50% inhibition of cell proliferation;

Our results show that both the ethanolic (EACE) and water (WACE) extracts are not toxic for HUVEC cells with (IC50 >> 2 mg/ml). Ethanolic and water extracts revealed an inhibition on HUVEC cells in a dose-dependent manner ($p = 0.01$) and ($p = 0.014$) respectively. Cis-platinium did not show any cytoselective effect. However, it was very toxic on HUVEC cells inducing an inhibition of cell growth (96%) at 10µM (Figure 30).

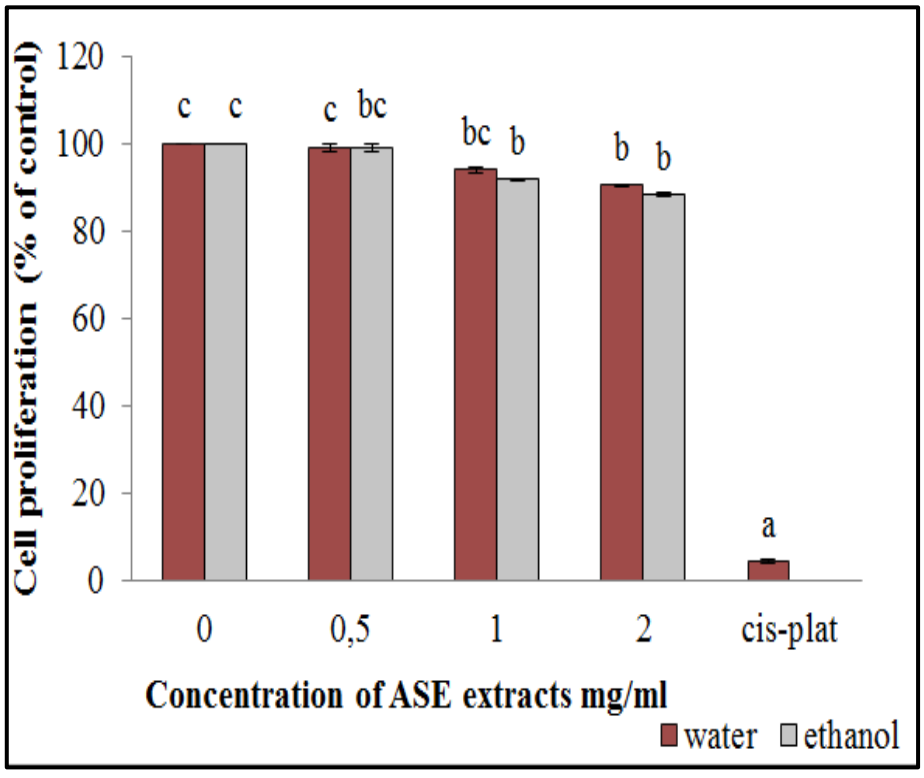

Figure 30: Effects of ASE crude extracts on cell proliferation of HUVEC cells

Each value represents (means ± SD, n=3, concentration 0 corresponding the untreated group). Means followed by different superscripts within a group are significantly different at P<0.05 according to Duncan's Multiple Range Test (DMRT)

III.6 Ethanolic and Water extract counteract HepG2 Proliferation

Figure 31 showed that survival of HepG2 cells was significantly reduced following incubation with ethanol (p =0.001) and water extracts (p= 00) (Cell proliferation was expressed as the mean percentages of viable cells relative to untreated cells). In addition, inhibitions of HepG2 cell proliferation by both extracts were dose-dependent. In particular 50 % of cell growth inhibition (IC50) was obtained when 0.7 mg/ml or 1.1 mg/ml of ethanolic or water extracts respectively, were added to the culture medium. In all cases, ethanolic extract was significantly more active than water extract (p=00). The maximum growth inhibition was obtained using Cis-plat (93.52 %) representing the positive control, followed by 2 mg/ml ethanolic extracts (82.5%) and 2mg/ml water extracts (68.63%).

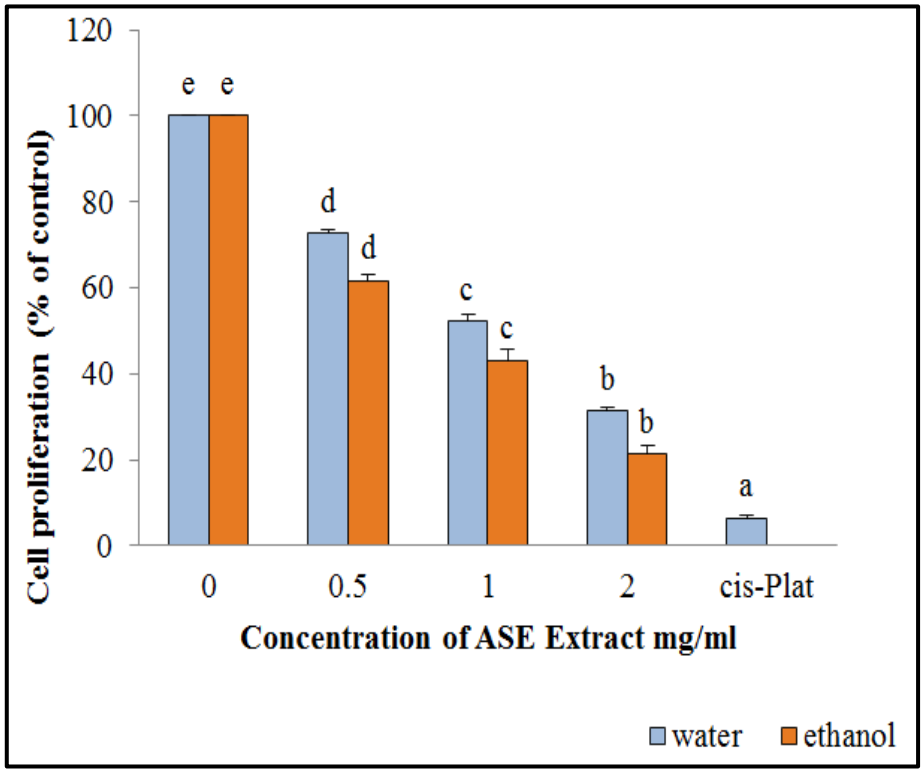

Figure 31: Effects of ASE crude extracts on cell proliferation of HepG2 cells

Each value represents means ± SD, n=3, concentration 0 corresponding the untreated group). Means followed by different superscripts within a group are significantly different at P<0.05 according to Duncan's Multiple Range Test (DMRT).

III.7 Ethanolic and water extracts influence the expression of apoptosis-related genes in HepG2 Cells

Figure 32 shows that HepG2 cultured in the presence of EACE or WACE exhibited a significant increase in Bax mRNA levels, as compared to untreated control cells in a concentration-dependent manner ($p<<0.05$). In the same cells Bcl-2 gene expression was down-regulated in a concentration-dependent manner ($p<<0,05$). Ethanolic extracts were more active on HepG2 cells than water extracts ($p=0.002$). In particular the maximum effect on both Bax and Bcl-2 genes was observed using the highest concentration (2 mg/ml) of ethanolic extracts (Figure 33).

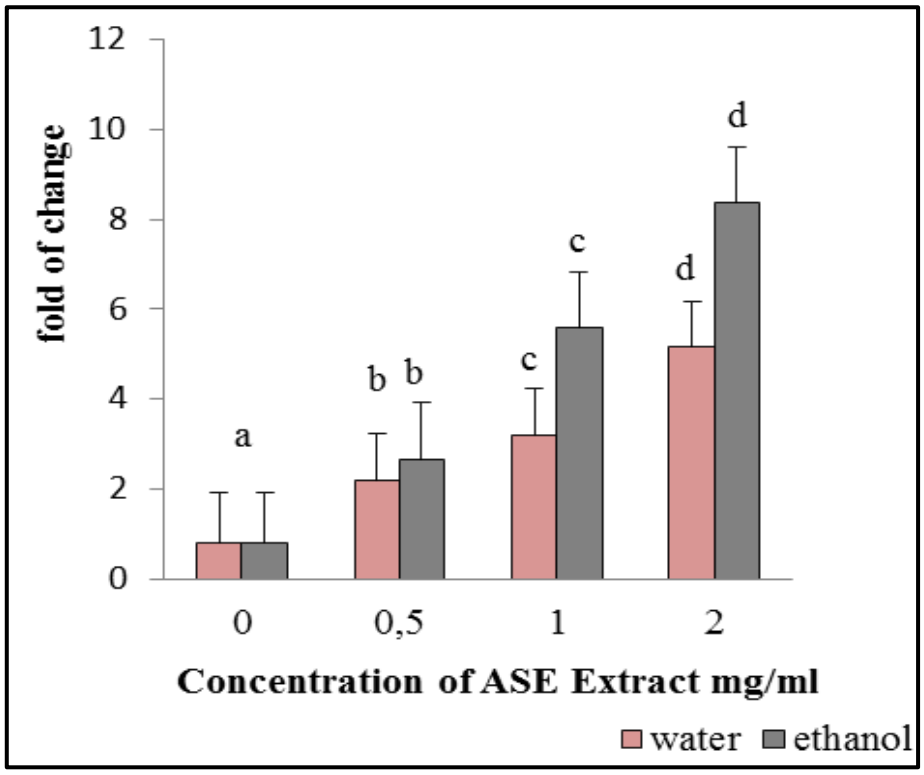

Figure 32: Effect of ASE crude extracts on Bax gene expression in HepG2 cells

The mRNA levels for each gene was expressed as fold of change ($2^{-\Delta\Delta Ct}$) relative to the untreated control (defined as 1) (mean ± SD; n = 3) and normalized to the Glyceraldehyde-3-Phosphate-Dehidrogenase (GAPDH). Means followed by different superscripts within a

group are significantly different at P<0.05 according to Duncan's Multiple Range Test (DMRT).

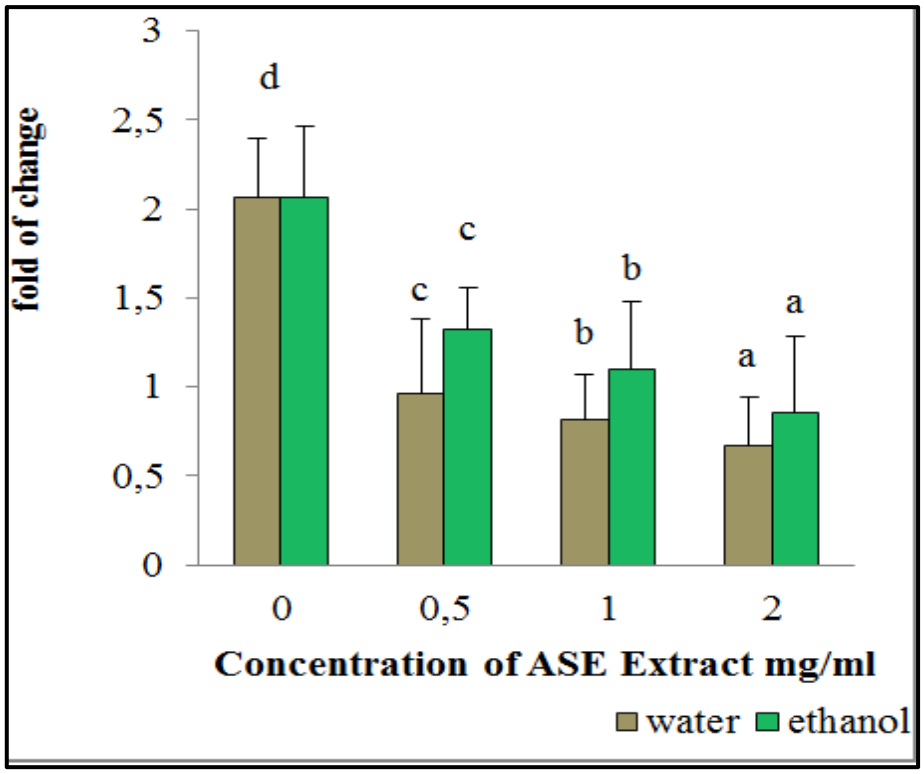

Figure 33: Effect of ASE crude extracts on Bcl-2 gene expression in HepG2 cells

The mRNA levels for each gene was expressed as fold of change ($2^{-\Delta\Delta Ct}$) relative to the untreated control (defined as 1) (mean ± SD; n = 3) and normalized to the Glyceraldehyde-3-Phosphate-Dehidrogenase (GAPDH). Means followed by different superscripts within a group are significantly different at P<0.05 according to Duncan's Multiple Range Test (DMRT).

III.8 Ethanolic and Water extracts influence MCF-7 Proliferation

As previously observed for HepG2, both EACE and WACE significantly inhibited MCF-7 proliferation (figure 34). In particular the IC50 for EACE and WACE of grapeleaves was 0.43 mg/ml, 0.71 mg/ml respectively. The ethanolic extract was significantly more active than water extract (p =00). The highest percentage of growth inhibition was obtained by Cis-plat (99.34%), followed by ethanolic (88.56 %) and water extracts (79.31%) (Figure 34). Results revealed that MCF-7 cells were more sensible than HepG2 cells.

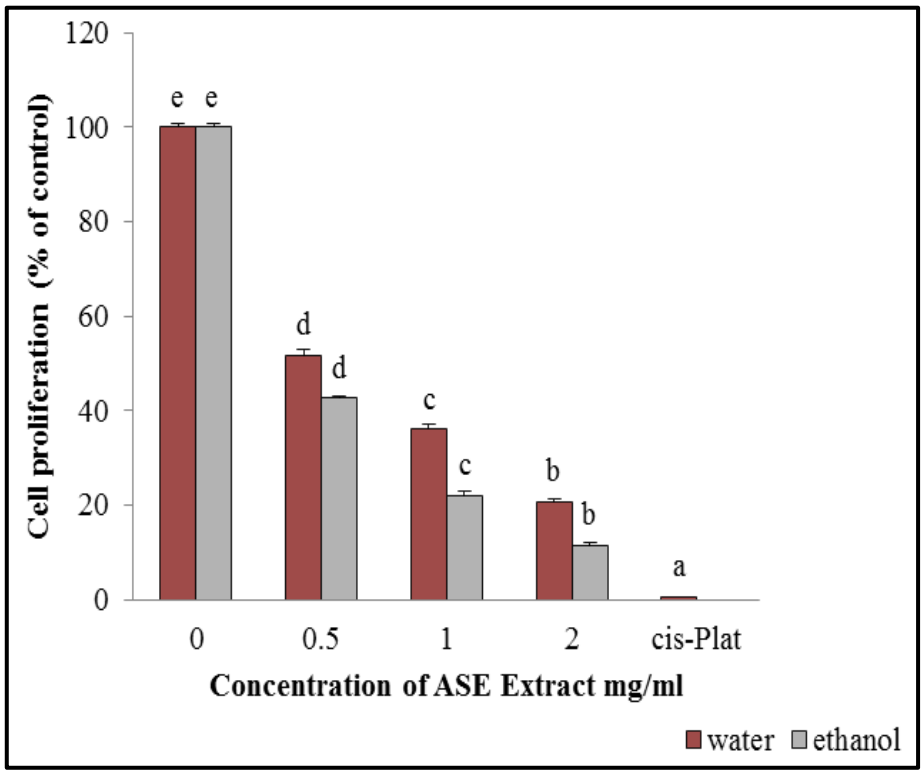

Figure 34: Effects of ASE crude extracts on MCF-7 cell proliferation

Each value represents means ± SD, n=3, concentration 0 corresponding the untreated group). Means followed by different superscripts within a group are significantly different at $P<0.05$ according to Duncan's Multiple Range Test (DMRT).

III.9 Ethanolic and Water extracts influence the expression of apoptosis-related genes in MCF-7

Ethanol and water extracts significantly modulated Bax and Bcl-2 mRNA expression levels in MCF-7 cells in a concentration-dependent manner (increased significantly Bax (p=00) and decreased significantly Bcl-2 (p= 0.002) (Figure 35-36). In particular, Bax gene expression was up-regulated, while Bcl-2 gene expression was down-regulated. The maximum effect was observed when the highest concentration (2 mg/ml) of ethanolic extracts or water extracts were added to the culture medium.

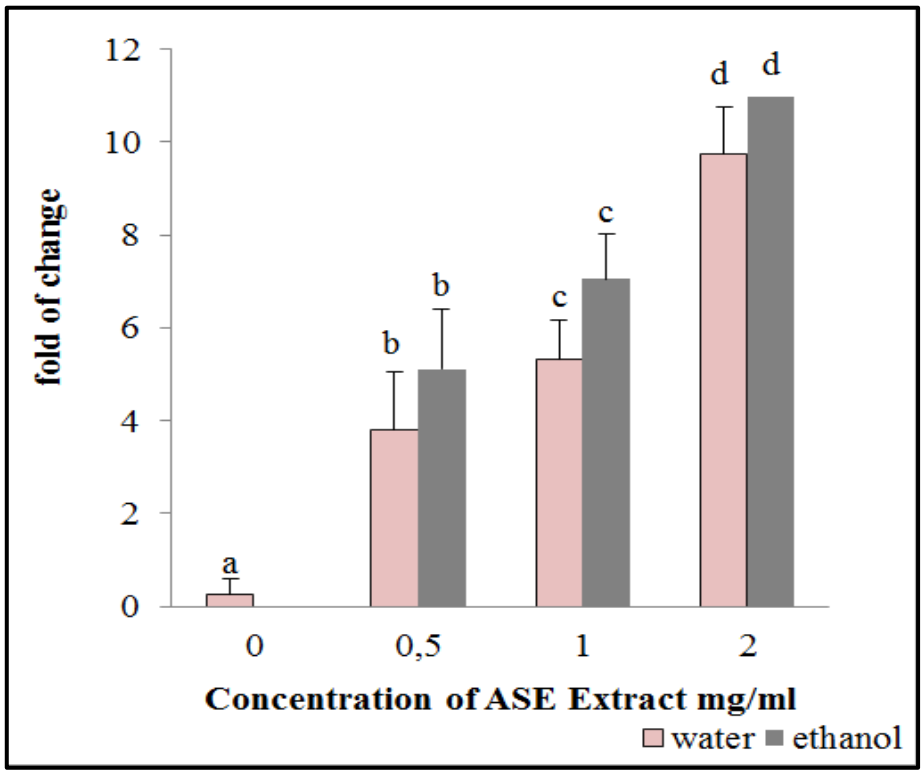

Figure 35: Effect of ASE crude extracts on Bax gene expression in MCF-7 cells

The mRNA level was expressed as fold of change ($2^{-\Delta\Delta Ct}$) as compared to untreated HepG2 cells (defined as 1) (mean ± SD; n = 3) and normalized to the Glyceraldehyde-3-Phosphate-Dehidrogenase (GAPDH). Means followed by different superscripts within a group are significantly different at $P<0.05$ according to Duncan's Multiple Range Test (DMRT).

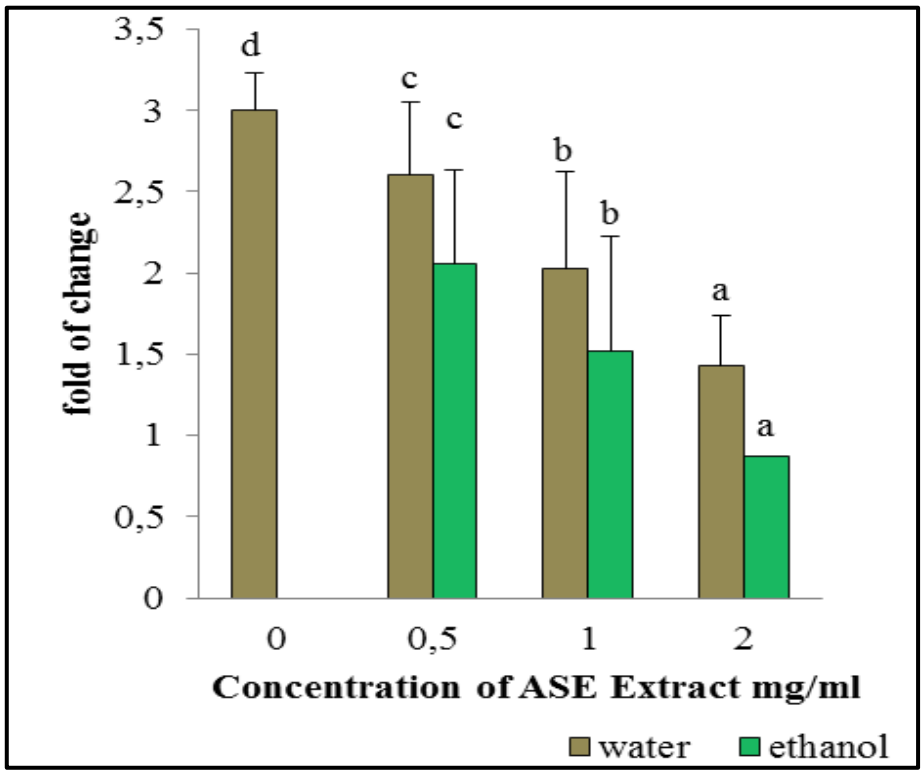

Figure 36: Effect of ASE crude extracts on Bcl-2 gene expression in MCF-7 cells

The mRNA level was expressed as fold of change ($2^{-\Delta\Delta Ct}$) as compared to untreated HepG2 cells (defined as 1) (mean ± SD; n = 3) and normalized to the Glyceraldehyde-3-Phosphate-Dehidrogenase (GAPDH). Means followed by different superscripts within a group are significantly different at $P<0.05$ according to Duncan's Multiple Range Test (DMRT).

Breast cancer is the most common cancer diagnosed among US women (excluding skin cancers) and is the second leading cause of cancer death among women after lung cancer. Approximately 252,710 new cases of invasive breast cancer and 63,410 cases of *in situ* breast carcinoma are expected to be diagnosed among US women in 2017. In addition, 40,610 women are expected to die from this disease in 2017 (Carol et al., 2017). Otherwise, Trends in hepatocellular carcinoma mortality rates have increased over recent decades in most countries. It is also the third cause of cancer death worldwide (Bertuccio et al., 2017).

Apoptosis can be activated through two major pathways, the mitochondria-dependent pathway and the death-receptor-dependent pathway. In the mitochondria-dependent signaling pathway, the Bcl-2 family of proteins is divided into two groups: suppressors of apoptosis (e.g., Bcl-2, Bcl-XL, Mcl-1) and activators of apoptosis (e.g., Bax, Bok, Hrk, Bad). The Bax/Bcl-2 ratio might represent a critical factor influencing cell behavior. Suppression of Bcl-2 promotes apoptosis in response to several stimuli, including anticancer drugs (Tan et al., 2009). Bax is a pro-apoptotic protein residing in the cytosol in an inactive form and translocating, after activation, to the mitochondria, where it plays an important role in mitochondria-mediated apoptosis. Activated Bax, either in homo-oligomeric form or as complex with other proteins, creates pores in the outer mitochondrial membrane, which leads to the leakage of ions, essential metabolites and cytochrome c from mitochondria to cytosol, thus promoting cell death (Farahmandzad et al., 2015). Our results demonstrated that grape leaves have an anti-proliferative effect on HepG2 and MCF-7 cells. EACE and WACE markedly inhibited HepG2 and MCF-7 cell viability.

In cells cultured with these extracts, the mRNA levels of the anti-apoptotic factor, Bcl-2, were downregulated, while the expression of the pro-apoptotic gene Bax, was significantly induced. Within this context, other authors have demonstrated that molecules as Diazaphenothiaznes exert an antiproliferative activity in MCF7 cells and C32 human amelanotic melanoma, by regulating Bax and Bcl-2 gene expression (Morak-Młodawska et al., 2015 and Morak-Młodawska et al., 2019).

Deepak et al. (2015) shows that desert plant extracts are able to induce apoptosis in HepG2 cells. They also describe an upregulation of Bax, Bad, cytochrome c, caspase-3, caspase-7, caspase-9 and poly (adenosine diphosphate-ribose) polymerase. Furthermore, the *Allium atroviolaceum* flower extracts was found to inhibit HepG2 cell growth, revealing a sub-G0 cell cycle arrest, changes in morphological features and annexin-V and propidium iodide positive staining, which correlates with Bcl-2 down-regulation and caspase-3 activity (Khazaei et al., 2017). Lu et al. (2011) report that injectable seed extracts from *Coix lacryma-jobi.L* induce apoptosis in HepG2 cells, with elevated and prolonged expression of caspase-8, which does not significantly influence the expression of Bcl-2. Moreover, Tan et al. (2009) report that quercetin can inhibit proliferation and induce apoptosis in HepG2 cells by decreasing the levels of surviving cells and Bcl-2 protein expression, and significantly increasing the protein levels of p53.

We found that the ethanolic crude extracts were able to induce a larger anti-proliferative effect as compared to the water crude extracts, which may be due to the different amount of phenols detected in the two different extracts. Nevertheless, further experiments are needed in order to understand if apoptosis could definitely explain the antiproliferative effects induced by the extracts tested in the present study.

Our extracts showed growth inhibition in MCF-7cells, confirming what has been previously described by other authors using different plant extracts. Blassan et al. (2016) report that *Rubus fairholmianus* root extracts inhibit MCF-7 cells growth via caspase 3/7-induced apoptosis. Reis et al. (2013) report that *Leccinum vulpinum* induces DNA damage, decreases cell proliferation and induces apoptosis in MCF-7 cells. Dikmen et al. (2011) report that pomegranate (*Punica granatum L.*), at some concentration, inhibits MCF-7 cell proliferation and induces increased expression of the pro-apoptotic gene Bax and decreased the expression of the anti-apoptotic gene Bcl-2. ASE extracts of grape leaves grown in Algeria were not cytotoxic for HUVEC cells. Atmaca and Bozkurt (2015) report that *Salvia triloba L.* extract has pro-apoptotic and anti-angiogenic effect in prostate cancer cell lines while being not cytotoxic for normal cells. Amirala et al. (2013) describe the pro-apoptotic potential of grape seeds extracts, confirmed by a significant inhibition of cell growth and viability in a dose- and time-dependent manner without inducing damage to HUVEC non-cancerous cells. Indeed, the bioactive phytochemicals, Honokiol and Magnolol contained in *Magnolia officinalis* and their derivatives show an antiproliferative effect on HepG2 cell proliferation while being unable to elicit any effect on fibroblasts (Maioli et al., 2018).

The literature strongly suggests that grape is a potential source of antioxidant, anticancer and cancer chemo-preventive phytochemicals. The other parts of the grapes, the skin and seeds, the whole grape by itself, grape-derived raisins and phytochemicals within the grapes have also been found to bear potential anticancer properties in various preclinical and clinical studies (Zhou et al., 2012).

The phenols, which are ubiquitous in almost all plant foods could decrease the risk of the occurrence of considerable number of diseases, particularly those related to aging and oxidative injury (cancer, cardiovascular diseases and neurodegenerative) (Hennebelle et al., 2004). While, low circulating levels of polyphenols (maximum, few µmol/L) compared to those of other endogenous antioxidants (GSH and acid uric) or exogenous (vit E as well as vit C) do not allow to envisage a direct antioxidant action of polyphenols in

the body. This, however, with the exception of the gastrointestinal tract where the polyphenols present in large quantities can act as scavengers of free radicals. Today at the level of organism, polyphenols are perceived as molecules “Signal” (Mornad et Milenkovic, 2014), that could stimulate multi-target modes of action. Many *in vitro* studies showed that flavonoids could affect their biological targets by modulating some enzymatic activities, gene expression or cell signaling, interacting with membrane or cell receptors, or via epigenetic regulations (Fraga et al., 2010). The diversity of these potential mechanisms of action explains the broad spectrum of activities flavonoids observed in-vivo, including anti-inflammatory activities, antioxidant and anti-angiogenic, anti-proliferative or pseudo-estrogenic (Mornad and Milenkovic, 2014)

Conclusion and Perspectives

Conclusion and Perspectives

Our study are conducted on series of experimental work divided on two parts; the first was *in vivo* in order to show the chemical composition of phenols of grape leaves Algerian Variety (GLAV) extract and its effects on hyperhomocysteinemia induced inflammatory endothelial damage in cardiovascular diseases.

Phenols were identified by using HPLC/DAD/ESI-MS analysis. The research of the anti-oxidant and anti-inflammatory effects was conducted on mice through 15 days. Results showed high levels of phenols, anthocyanins, flavonols and *trans*-caftaric acid in GLAV. The plasma hs-CRP and homocysteine levels were elevated significantly ($p < 0.05$) however the glutathione reduced significantly ($p < 0.05$) after the administration of L-methionine in high doses to mice. This was associated with the desquamation of endothelium and muscular lysis with transformation of spindle nuclei to oval nuclei; this is due to the angiotoxic action of homocysteine on the aorta. These changes were not observed in mice treated with L-methionine plus the antioxidant and anti-inflammatory extract of GLAV.

These results proved the antioxidant and anti-inflammatory effects of the GLAV on hyperhomocysteinemia induced inflammatory endothelial damage in cardiovascular diseases.

The second part was achieved *in vitro* toward to measure the polyphenol, anti-oxidative and anti-proliferative properties of water and ethanol Accelerator Solvent Extractor (ASE) crude extracts from GLAV.

The amount of total phenols was determined using the modified Folin-Ciocalteu method, antioxidant activities were evaluated by the 2,2-diphenyl-l-picrylhydrazyl free radical ($DPPH^{\cdot}$) method and •OH radical scavenging using electron paramagnetic resonance (EPR) spectroscopy methods. Cell proliferation of HepG2 hepatocarcinoma, MCF-7 human breast cancer cells and vein human umbilical (HUVEC) cells, as control for normal cell growth, was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay (MTT). Apoptosis related genes were determined by measuring Bax and Bcl-2 mRNA expression levels.

Accelerator solvent extractor yield did not show significant difference between the two solvents (ethanol and water) ($p > 0.05$). Total phenolic content of water and ethanolic extracts was (55.41±0.11) and (155.73 ± 1.20) mg of Gallic acid equivalents/g of dry weight, respectively. Ethanolic extracts showed larger amounts of total phenols as

compared to water extracts and interesting antioxidant activity. HepG2 and MCF-7 cell proliferation decreased with increasing concentration of extracts (0.5, 1, and 2 mg/ml) added to the culture during a period of 1–72 h. In addition, the expression of the pro-apoptotic gene Bax was increased and that of the anti-apoptotic gene Bcl-2 was decreased in a dose-dependent manner, when both MCF-7 and HepG2 cells were cultured with one of the water and ethanolic extracts for 72 h. None of the extracts elicited toxic effects on vein umbilical HUVEC cells, highlighting the high specificity of the antiproliferative effect, targeting only cancer cells.

Our results suggested that ASE crude extract from GLAV represents a source of bioactive compounds such as phenols, with potential antioxidants activity, disclosing a novel antiproliferative effect affecting only HepG2 and MCF-7 tumour cells.

In perspectives more studies in this area are required further to find:

1. Optimizing and screening the exact active elemental composition of our extracts;
2. New ways and new efficient molecules from *Vitis vinifera* leaves to treat degenerative diseases and to slow the aging process induced by oxidative intermediate products and other pro-inflammatory components;
3. The exact manner action of our extracts on the cell proliferation;
4. Manner of preparing drugs or dietary supplements counteracting distinctive cancer cells proliferation.
5. Exploiting -Omics technologies in order to discover the roles, relationships, and the exact actions of the various types of molecules present in the GLAV on cells of an organism.

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Appendices

Appendices

1. Composition of diet taken by mice during 15 days of treatment (OFFICE National de l'Aliment du Bétail) (ONAB).

Food material	Amount in g/kg	Diet Percentage %
Corn	620	62
Soy	260	26
Phosphate	16	1,6
Limestone	9	0,9
Cellulose	10	1
Minerals	10	1
Vitamins	10	1

2. Evaluation of the plant extracts anti-oxidant activity

The anti-oxidant activity was measured by spectrofiguremetric determination of glutathione from liver's homogenate following the method of Weckbeker et al, 1988 (Rahman et al., 2006).

At the end of the experience for the Immunostimulatory activity the animals were sacrificed and the liver and spleen dissected and weighted immediately in the wet state.

2.1 Preparation of the homogenate

The weight of 0,5g of the liver was homogenized in 2 ml of TBS (Tris 50 mM, NaCl 150 mM, pH 7.4). Then the homogenates were centrifuged at 9000 g for15 min at 4˚C after that the supernatant was used for determination of glutathione reduced (GSH).

2.2 GSH dosage

The glutathione reduced content in the liver was measured spectrophotometrically by using 5,5′-dithiobis-(2 nitrobenzoic acid) (DTNB) as a coloring reagent. The experimental procedure for the glutathione dosage is the following:

- 0.8 ml of the homogenate was added to 0.2 ml of the sulfo-salicylique acid (0.25%) solution, Then the mixture was incubated in an ice bath for 15 min.
- Next a centrifugation at 1000 tours/min for 5 min was realized. After that, 0.5 ml of the supernatant and 1 ml of the buffer Tris-EDTA (PH 9.6) were added to 0.025 ml of DTNB of 0.01 M and after 5 min the optical density was measured at 412 nm.
- The glutathione concentration was calculated by the following formula :

$$GSH\ (\text{nmol}\frac{\text{GSH}}{\text{mg}}\text{protein}) = \frac{DO \times 1 \times 1.525}{13100 \times 0.8 \times 0.5 \times \text{mg protein}}$$

⇒ DO: Optical Density.

⇒ 1: total volume of the protein decomposition solutions (0.8 ml the homogenate + 0.2 sulfo-salicylique acid).

⇒ 1.525: volume of the total solutions used for the GSH dosage at the supernatant level (0.5 ml supernatant + 1 ml Tris-EDTA + 0.025 ml DTNB).

⇒ 13100: group –SH absorbance coefficient at 412 nm.

⇒ 0.8: homogenate volume.

⇒ 0.5: supernatant volume.

The proteins concentration was determinated by method of Bradford (1976) by adding 0.1 ml of the homogenate with 5 ml of the Bradford reagent and after 5 min the optical density was measured at 595 nm. The proteins concentration was calculated by a comparison to the BSA calibration line realized in the same conditions.

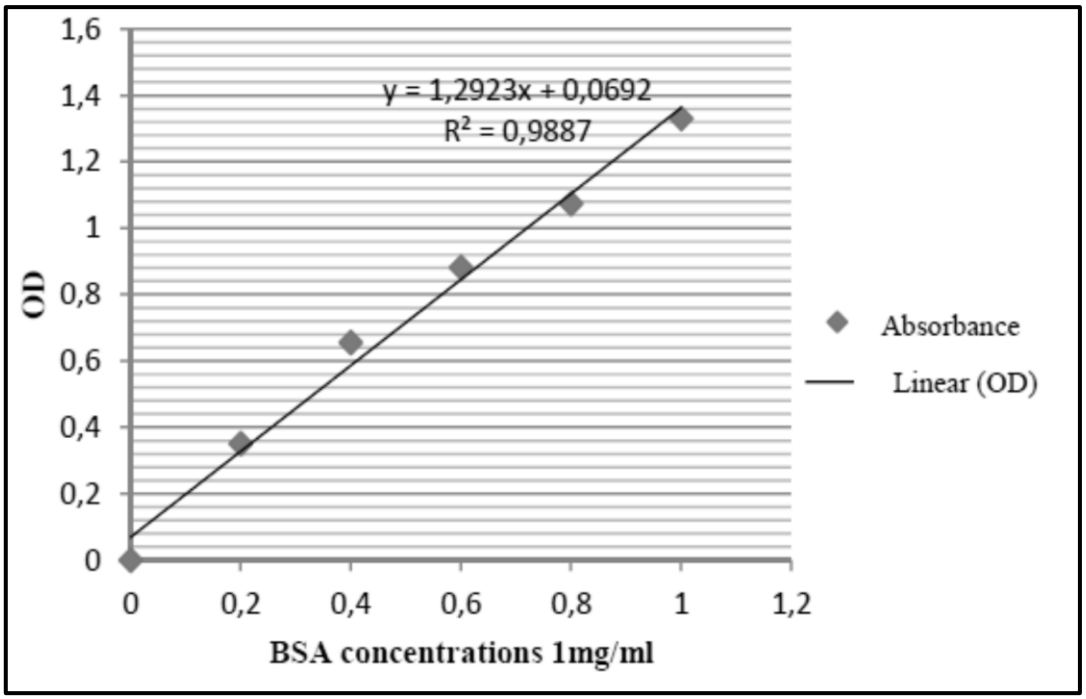

Figure 37: Calibration curve

3. Preparation of cell culture medium

The cell culture medium was prepared by using, 200 ml of SIGMA MEM (Minimal Essential Medium), 20 ml of FBS (Fetal Bovine Serum) (10%), 4 ml of antibiotic made of penicillin and streptomycin, 2 ml of glutamine and 2 ml of SIGMA MEM nonessential amino acid.

4. Trypsinization and cell counting

Human breast cancer cell line (MCF7), hepatocarcinoma cells (HepG2), normal Human Umbilical Vein Endothelial Cell (HUVEC) from one T75 flask were used in this part of the study.

- First, the medium was removed and the flask was washed by 2 ml of Dulbecco's Phosphate-Buffered Saline (DPBS) (gibco® by Life Technologies™);
- After removing the DPBS, 1.5 ml of trypsin (gibco® by Life Technologies, cascade biologics™) was added to the flask (cells + trypsin);
- Then incubated for 5 min in CO_2 incubator (Thermo scientific "FORMA STERI-CYCLE");
- After that, 4.5 ml of medium was added to flask (cells + trypsin + medium) and with a smooth pipetting to mix the solutions which then had been transferred in conic tubes with 3 ml of DPBS used also to wash the cells from the flask. Then the tubes transferred for 5min/300g centrifugation (Thermo scientific SL40);
- After the centrifugation, the supernatant was removed and the cells were solubilized in 2 ml of the medium;
- The next step was to know the number of cells in the 2 ml of cell solution. For that objective, 10 µl of trypan bleu was added to 10 µl of the cell solution;
- Then, 10 µl of the mixture wastransferred to each side of Countess™ cell counting chamber slides. The last step was the cellcounting using Invitrogen™ "Contess automated cell counter".

5. The RNA extraction was realized following Trizol protocol:

- 500µl of Trizol was added to each eppendorf with 100µl of chloroform;
- Agitated for 15 sec with vortex;

- Incubated for 10 min;
- Then, the solution was centrifuged for 15min (4°C, 12000g);
- The supernatant was recuperated (the transparent phase);
- Added with 250 µl of isopropanol; (shake and put for 10 min in ice);
- Then the tubes were centrifuged for 10 min (4°C, 12000g);
- After removing the supernatant, 500µl of ethanol was added and then centrifuged for 5min (7500g);
- Next, the ethanol was removed (wait ≈ 5min to evaporate all the ethanol) and 20µl of water was added.

6. Passage from T25 flask to T75 flask and from T75 to T75 flask

The passage of the cancer cells from a T25 flask to a T75 flask was made by a trypsinization protocol.

- First the medium was removed and the flask was washed with 2 ml of DPBS (4 ml for T75-T75).
- Then, the DPBS was removed and 1 ml of the trypsin (1.5 ml for T75-T75) was added to the cells and incubated in CO_2 incubator for 5 min.
- After that, 3 ml of the medium was added to the flask (4.5 ml for T75-T75) and with a smooth pipetting the cells were separated and the mix was then transferred to a conic tube with 2 ml of DPBS (3 ml for T75-T75) used to wash the flask and then the solution was centrifuged for 5min/300g.
- After taking out the supernatant 4 ml of the medium was added to the cells (by a smooth pipetting). At the end, 2 ml of the medium and cells solution was transferred to two T75 flasks filled with 7 ml of the medium each.

7. Protocol for Cryoconservation

The medium of the cryoconservation is made of: 50% meduim (used in the cell culture), 40% FBS and 10% DMSO.

- First the medium was removed and the flask was washed with 2 ml of DPBS.

- Then, the DPBS was removed and 1.5 ml of the trypsin was added to the cells and incubated in CO_2 incubator for 5 min.
- After that, 4.5 ml of the medium was added to the flask and with a smooth pipetting the cells were separated and the mix was then transferred to a conic tube with 3ml of DPBS used to wash the flask and then the solution was centrifuged for 5min/300g.
- After removing the supernatant, the cells were re-suspended in the cryoconservation medium.
- The cells in 1 T75 flask were conserved in 3 cryotubes of 1 ml.
- Cryotubes = 18 ml of cryoconservation medium.
- Medium + DMSO = 50% + 10% = 9ml + 1.8 ml = 10.8 ml.
- The cells in each flask were re-suspended in 1.2 ml of FBS then400 µl of that solution was transferred in 1 cryotube with 600µl from the 10.8 ml of the medium + DMSO solution.

8. RNA quantities MCF-7

SAMPLES	1ST READ ng/µL	2ND READ ng/µL	AVERAGE ng/µL	µL (200 ng in x µL)	µL of DPCW
W1/1	305,3	311,9	308,6	0,64808814	14,35191186
W1/2	433,4	432,1	432,75	0,462160601	14,5378394
W1/3	570,3	546	558,15	0,358326615	14,64167339
W2/1	535,9	480	507,95	0,393739541	14,60626046
W2/2	346,8	353,5	350,15	0,571183778	14,42881622
W2/3	233,8	239,3	236,55	0,845487212	14,15451279
W3/1	847,2	844,9	846,05	0,236392648	14,76360735
W3/2	289,1	349	319,05	0,626860994	14,37313901
W3/3	865,8	855,1	860,45	0,232436516	14,76756348
ET1/1	89,5	89,8	89,65	2,230897936	12,76910206
ET1/2	88,4	88,4	88,4	2,262443439	12,73755656
ET1/3	153,7	153	153,35	1,304206065	13,69579394

ET2/1	29,3	29,6	29,45	6,791171477	8,208828523
ET2/2	164,9	175,6	170,25	1,174743025	13,82525698
ET2/3	248,9	251,1	250	0,8	14,2
ET3/1	152,9	150,7	151,8	1,317523057	13,68247694
ET3/2	222,8	223,4	223,1	0,896458987	14,10354101
ET3/3	673,7	651,5	662,6	0,301841232	14,69815877
(+)/1	290,4	302,9	296,65	0,67419518	14,32580482
(+)/2	88,9	74	81,45	2,455494168	12,54450583
(+)/3	312,1	312	312,05	0,640922929	14,35907707
(-)/1	402,3	405,9	404,1	0,494926998	14,505073
(-)/2	254,5	264,8	259,65	0,770267668	14,22973233
(-)/3	227,8	227,8	227,8	0,877963126	14,12203687

9. RNA quantities HEGP2

SAMPLES	**1ST READ**	**2ND READ**	**AVERAGE**	**µL (200 ng in x µL)**	**µL of DPCW**
W1/1	2289	2201,6	2245,3	0,089074957	14,91092504
W1/2	1644,8	1689,1	1666,95	0,119979603	14,8800204
W1/3	1851,2	1877,9	1864,55	0,107264487	14,89273551
W2/1	1305,1	1270,6	1287,85	0,155297589	14,84470241
W2/2	732,4	726,1	729,25	0,274254371	14,72574563
W2/3	1390,3	1365,6	1377,95	0,145143147	14,85485685
W3/1	674,8	672,3	673,55	0,296934155	14,70306585
W3/2	1383,4	1370,5	1376,95	0,145248557	14,85475144
W3/3	1006	1011,3	1008,65	0,198284836	14,80171516
ET1/1	430,9	472	451,45	0,443016945	14,55698305
ET1/2	409	436,8	422,9	0,472925041	14,52707496
ET1/3	816,3	812,6	814,45	0,245564491	14,75443551
ET2/1	296,7	297,1	296,9	0,673627484	14,32637252

ET2/2	760,7	759,1	759,9	0,263192525	14,73680747
ET2/3	279,4	273,5	279,4	0,715819613	14,28418039
ET3/1	1546	1451,7	1498,85	0,133435634	14,86656437
ET3/2	1137,2	1139,4	1138,3	0,175700606	14,82429939
ET3/3	919,2	918,8	919	0,217627856	14,78237214
(+)/1	938,6	907	907	0,220507166	14,77949283
(+)/2	1303,4	1287,6	1295,5	0,154380548	14,84561945
(+)/3	1071,9	1073,1	1072,5	0,186480186	14,81351981
(-)/1	651,6	631,7	641,65	0,311696408	14,68830359
(-)/2	135,8	145	140,4	1,424501425	13,57549858
(-)/3	557,7	502,8	530,25	0,377180575	14,62281942

10. Cancer plate of MCF-7 and HEGP2

	1	2	3	4	5	6	7	8	9	10	11	12	
A	W1 +MCf7	W1 +MCf7	W1 +MCf7				W1 +Hegp2	W1 +Hegp2	W1 + Hegp2	MC7	MC7	MC7	A
B	W2+MCf7	W2+MCf7	W2+MCf7				W2+ Hegp2	W2+ Hegp2	W2+ Hegp2P	Hegp2	Hegp2	Hegp2	B
C	W3+MCf7	W3+MCf7	W3+MCf7				W3+ Hegp2	W3+ Hegp2	W3+ Hegp2	+	+	+	C
D	W4+MCf7	W4+MCf7	W4+MCf7				W4+ Hegp2	W4+ Hegp2	W4+ Hegp2	+	+	+	D
E	ETH1+MCf7	ETH1+MCf7	ETH1+MCf 7				ETH1+ Hegp2	ETH1+ Hegp2	ETH1+ Hegp2	DMSO	DMSO	DMSO	E
F	ETH2+MCf7	ETH2+MCf7	ETH2+MCf 7				ETH2+ Hegp2	ETH2+ Hegp2	ETH2+ Hegp2				F
G	ETH3+MCf7	ETH3+MCf7	ETH3+MCf 7				ETH3+ Hegp2	ETH3+ Hegp2	ETH3+ Hegp2				G
H	ETH4+MCf7	ETH4+MCf7	ETH4+Mcf 7	1			ETH4+ Hegp2	ETH4+ Hegp2	ETH4+ Hegp2				H
		2	3	4	5	6	7	8	9	10	11	12	

W; WATER EXTRACT 1, 2, 3, ARE THE CONCENTRATIONS, ETH; ETHANOL EXTRACT 1, 2, 3 ARE THE CONCENTRATIONS HEGP2 $1.5*10^4$ MCF-7 $1.5*10^4$ Positive control Cis-plat.

Papers

Paper 01

THE EFFECTS OF GRAPE LEAVES EXTRACT ON HYPERHOMOCYSTEINEMIA INDUCED INFLAMMATORY ENDOTHELIAL DAMAGE IN CARDIOVASCULAR DISEASES

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Abstract. This study was designed to detect the anti-oxidant and anti-inflammatory effects of phenolic compounds in grape leaves Algerian variety (GLAV) on endothelial damage. The phenolics were identified by using HPLC/DAD/ESI-MS analysis. The research of the anti-oxidant and anti-inflammatory effects was conducted on mice through 15 days. Results showed high levels of phenols, anthocyanins, flavonols and *trans*-caftaric acid in GLAV. The plasma hs-CRP and homocysteine levels were elevated significantly ($p < 0.05$) however the glutathione reduced significantly ($p < 0.05$) after the administration of L-methionine in high doses to mice. This was associated with the desquamation of endothelium and muscular lysis with transformation of spindle nuclei to oval nuclei; this is due to the angiotoxic action of homocysteine on the aorta. These changes were not observed in mice treated with L-methionine plus the antioxidant and anti-inflammatory extract of GLAV. So, the study proved the antioxidant and anti-inflammatory effects of the GLAV on hyperhomocysteinemia induced inflammatory endothelial damage in cardiovascular diseases.
Keywords: *grape leaves, phenols, total Hyc, hs-CRP, GSH, endothelial damage*

Introduction

Hyperhomocysteinemia is defined as an abnormally high plasma homocysteine (Hcy) concentration after an oral Methionine load (Van Den Berg et al., 1995). It is a factor of risk for premature cardiovascular disease (Williams et al., 2002). Hence, it is one of the major pathogenic factors of atherosclerosis (Boldyrev et al., 2009). Besides its detection in all inflammatory diseases, hyperhomocysteinemia has been reported in other sicknesses like: type 2 diabetes, chronic kidney disease and cancer (Wu, 2008; Falvoa, 2007), and Alzheimer (Morris et al., 2001). It should be mentioned that hyperhomocysteinemia is not produced only by inflammation, but also by oxidative stress generated by high plasma homocysteine, which can cause a hyperhomocysteinemia induced inflammation (Jacobsen, 2000).

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The relationship between hyperhomocysteinemia and cardiovascular disease is highlighted by the deficiency of the cystathionine beta-synthase (CβS) enzyme, which is deficient during homocysteinuria (Flemming et al., 2010). In most cases, hyperhomocysteinemia is a result of deficiency of the vitamins B6, B12, folate, or a combination of them (Chiang et al., 2005). These vitamins are essential co-factors of the key enzymes of the Homoysteine's metabolism. Moreover, some drugs such as fibrates, antiepileptic, methotrexate, theophylline, metformin, and other substances like nicotinic acid can also cause hyperhomocysteinemia (Stalder et al., 2010). Homocysteine acts directly on endothelial and damaged vessel wall through generating an oxidative stress, and stimulating a pro-coagulant and pro-inflammatory state of blood components (Bernardo et al., 2004) is the most accepted hypothesis about Hcy's action in cardiovascular disease.

Several studies have demonstrated that correcting the plasma deficiency of folic acid and vitamin B12 decreases or makes hyperhomocysteinemia disappear (Rigaud, 1999). But in this study, the focus is on natural antioxidants, especially plants traditionally used in folk medicine and precisely GLAV (*Vitis vinifera* L leaves).

Vitis vinifera L. is a widespread crop in Algeria. In 2000, following the Algerian agriculture ministry, vineyards occupied an area close to 56,500 ha. *Vitis vinifera* leaves have been traditionally used as food or as medications all over the world. The leaves are used to treat hypertension, diarrhea, hemorrhage and varicose veins, inflammatory disorders, and reduce blood glucose levels in diabetics (Dani et al., 2010).

The aims of the study is identifying the phenolic compounds (Anthocyanins and non anthocyanins) by using HPLC-DAD/ESI-MS, and measuring the total Hyc, the plasma hs-CRP and the concentration of the GSH to estimate the antioxidant and anti-inflammatory effect of the GLAV on the inflammation induced by Hyperhomocysteinemia. By the end, the aorta histology had been examined in order to confirm the angiotoxic action of homocysteine and the effect of GLAV on the aorta.

Materials

Plant material

The plant material was collected from the leaves of fully matured grape (*Vitis vinifera* L.) in August, from Media Algeria. Leaves were rinsed in tap water and freeze-dried. Afterwards, they were crushed with a blender for 5 min and the resulting powder was collected and stored in the dark at 20 °C until needed.

Chemicals

All the used solvents and the formic acid of HPLC-grade (from Merck, Darmstadt, Germany). The water was purified by a milli-Qplus system from Millipore (Milford, MA, USA). All the employed Reagents were of analytical grade, purchased from Carlo Erba (Milan, Italy). The standards of HPLC-grade[malvidin-3-glucoside (n° 04288)], the 5,5′-dithiobis-(2 nitrobenzoic acid) DTNB and all the rest of chemicals were purchased from Sigma-Aldrich, Inc. 0.45 μm Polytetrafluoroethylene (PTFE) syringe membrane filters was purchased From Waters Co. - Milford, MA, USA.

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Methods

Analysis of anthocyanins and non-anthocyanin phenolic compounds of GLAV by reverse phase liquid chromatography-diode array detection/electrospray mass spectrometry (RPLC-DAD/ESI-MS)

Sample preparation

Three grams of powdered leaves were extracted three times at 1:2 (weight/volume) (w/v) ratio with cold methanol: HCl (1000:1 (volume/volume) (v/v) by using an ultra turrax (Ultra Turrax-Tube Drive, BM-G-ball-mill tube, IKA, Germany) with 10/CS glass balls, for 3 min of each sample. After the extraction, centrifugation took place (1600 *g*, 15 min, 4 °C), the supernatant was collected and stored on ice. The pellet was re-subjected to subsequent extraction, and a final volume of 14.5 ml was collected. The methanol/HCl extract was first filtered through the 0.45 μm membrane PTFE, and then it was exposed to Speed Vac concentration (SC250P1-250, Thermo Fisher Scientific Inc, Waltham, MA, USA) at 20 °C until dryness. Next, the residue was brought to a final volume of 10 ml by adding formic-acidified (pH 3.2) MQ water and kept at -20 °C until analysis. Discarding the pellets, the extraction was performed in triplicate **(**Kammerer et al., 2004**)**.

HPLC-DAD-ESI-MS characteristics and protocols

HPLC-DAD and HPLC-MS apparatus

The reverse phase liquid chromatography (RPLC) was performed according to Villiers et al. (2004) and Kammerer et al. (2004) with slight changes using a High Performance Liquid Chromatography system consisting of Hewlett-Packard series 1100 L equipped with a Diode Array Detector (DAD) operated by a HP 9000 workstation (Agilent Technologies, Palo Alto, CA, USA). The HPLC-MS system was equiped with HPLC-DAD instrument coupled to a quadripole mass spectrometer HP 1100 MSD electrospray interface (ESI) (Agilent Technologies, Palo Alto, CA, USA). The Separation had been occurred on a reverse-phase Waters Nova-Pak C_{18} column [150 mm × 3.9 mm, 4 μm] for anthocyanins analysis and a reverse-phase Waters Nova-Pak C_{18} column [300 mm × 3.9 mm, 4 μm] for non-anthocyanin phenols, and both had been kept at 26 °C with a pre-column of the same phase.

DAD and ESI-MS parameters

The non-anthocyanin phenols DAD was performed according to Dobes et al. (2013) with slight changes from 220 to 380 nm and ESI-MS parameters were: drying gas (N_2) at 350 °C with a 10 L/min flow, nebulizer pressure at 380 Pa (55 psi), and capillary voltage of 4000 V. The ESI scanned the mass from *m*/*z* 100 to 3000, employing a fragmentator voltage gradient of 100 V from 0 to 200 *m*/*z* and 200 V from 200 to 3000 min.

On the other hand, The anthocyanins DAD was performed from 260 to 600 nm, and ESI-MS parameters were: drying gas (N_2) at 350 °C with a 10 L/min flow, nebulizer pressure at 380 Pa (55 psi), and capillary voltage of 4000 V. The ESI scanned the mass from *m*/*z* 100 to 1500, employing a fragmentator voltage gradient of 100 V from 0 to 17 *m*/*z* and 120 V from 17 to 55 min.

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Elution parameters

Whereas, the elution of non-anthocyanin phenols was performed at a 0.7 mL/min gradient flow of solvent 'A' and 'B'. The former was a combination of water/acetic acid (98:2, v/v), and the latter constituted of water/acetonitrile/acetic acid (78:20:2, v/v/v). A linear gradient started with 0% of 'B' and 100% of 'A', and then reached 80% of 'B' and 20% of 'A' after 55 min. After that, slight linear increase of 'B', from 80 to 90%, between 55 to 57 min, was observed, then it remained isocratic at 90% of 'B' and 10% of 'A'. Next, the process increased linearly from 90 to 95% of 'B' during 10 min. Finally, and still in a linear way, it reached 100% of 'B' at 90 min. The column was washed with Methanoland re-equilibrated from 90 to 120 min. The volume of the injected leaves extract was 15 µL.

Eluent of anthocyanins was performed at a 0.8 mL/min gradient flow of solvent 'A' and 'B'. The former was a combination of water/formic acid (90:10, v/v), and the latter constituted of water/methanol/formic acid (45:45:10, v/v/v). A linear gradient started with 15% of 'B' and 85% of 'A', then it reached 80% of 'B' and 20% of 'A' after 30 min.

After that, the process continued in an isocratic way, from 30 to 45 min, with 80% of 'B'. The column was then rinsed with methanol and re-equilibrated from 43 to 75 min. The injected volume of leaves extract was150 µL, and formic acid was employed as a pH modulating agent in order to optimise the anthocyanins detection by maximizing the absorption in the λ 520 nm region.

HPLC/DAD/ESI-MS identification of anthocyanins

The identification of malvidin 3-glucoside and cyanidin 3-glucoside was performed by comparing the results of commercial standards (Sigma-Aldrich) with the positive ion mass spectra achieved from the ESI-MS (retention time (t_R), UV λ_{max}, and MS^n) of the leaves extract, while other anthocyanins were identified by comparing the ESI-MS attained results against the ones available in the literature (*Table 1*). The flavonols were identified (glucose/galactose and glucuronide derivatives) under ESI-MS. This latter was achieved according to the molecular and fragment ions $[M\text{-H-}162]^-$ (quercetin-3-galactoside/glucoside) and $[M\text{-H-}176]^-$ (quercetin-3-glucuronide) (*Figs. 1* and *2*). In addition, the identity of all other constituents was validated by comparing the attained retention times (t_R), UV λ_{max}, and MS^n of peaks from the leaves extract with those reported in the literature (*Table 1*).

Compound quantitative analysis by HPLC/DAD

The quantification of anthocyanins in the leaves extract was obtained by measuring peak areas at 530 nm and taking into account the external standard calibration curve of malvidin-3-glucoside, which was measured at 524 nm. The concentrations were expressed as 'µg malvidin-3-glucoside equivalents/g of freeze-dried leaves'. The contribution of single anthocyanins was calculated and expressed in % of the total anthocyanins content.

The quantification of flavonols was performed according to the peaks attained at 340 nm and calculating the concentrations as 'µg quercetin or kaempherol equivalents/g of freeze-dried leaves' according to external standard calibration curve of quercetin and kaempherol. The contribution of single flavonols was calculated and expressed as % of the total flavonol content.

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Quantification of *trans*-caftaric acid was based on an external standard calibration curve carried out at 340 nm and expressed as 'µg *trans*-caftaric acid/g of freeze-dried leaves'.

The external standard calibration curves for malvidin-3-glucoside, quercetin, kaempherol and *trans*-caftaric acid, which were performed in duplicate by using five dilutions within linearity and an R^2 values, were 0.98, 0.97, 0.98 and 0.99 respectively.

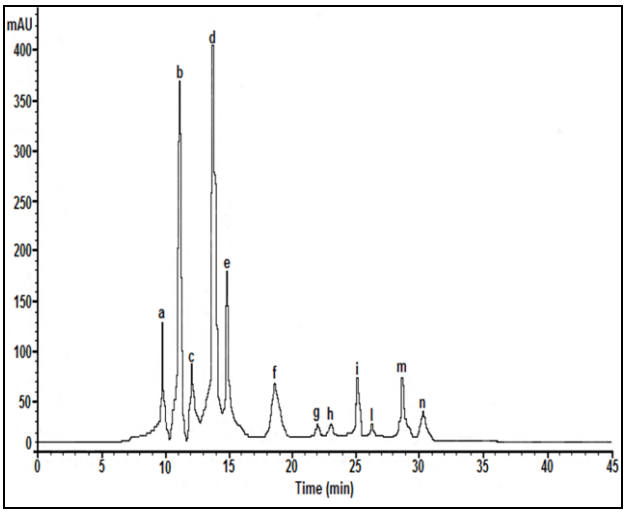

Figure 1. *HPLC-DAD Chromatogram at 530 nm of anthocyanin compounds from a Vitis vinifera L. leaves methanol extract. (a) delphinidin 3-glucoside; (b) cyanidin 3-glucoside; (c) petunidin 3-glucoside; (d) peonidin 3-glucoside; (e) malvidin 3-glucoside; (f) cyanidin 3-(6-p-acetyl) glucoside; (g) peonidin 3-(6-acetyl) glucoside; (h) delphinidin 3-(6-p-coumaroyl) glucoside; (i) cyanidin 3-(6-p-coumaroyl) glucoside; (i) petunidin 3-(6-p-coumaroyl) glucoside; (l) petunidin 3-(6-p-coumaroyl) glucoside; (m) peonidin 3-(6-p-coumaroyl) glucoside; (n) malvidin 3-(6-p-coumaroyl) glucoside*

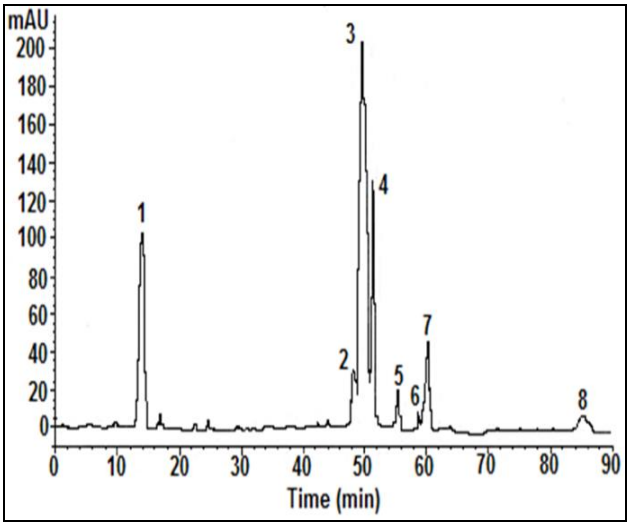

Figure 2. *HPLC-DAD Chromatogram at 340 nm of non-anthocyanin compounds from a Vitis vinifera L. leaves methanol extract. (1) trans-caftaric acid; (2) Quercetin-3-O-galactoside; (3) quercetin 3-0-glucuronide; (4) quercetin 3-0-glucoside; (5) kaempferol 3-0-galactoside; (6) kaempferol 3-0-glucuronide; (7) kaempferol 3-0-glucoside; (8) quercetin*

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Effect of GLAV on the inflammation induced by hyperhomocysteinemia

We are following the same protocol (the same conditions and diet) used by Benmbarek et al. (2013) but with a change in concentration of methionine and treatment period.

Animals and diets

Twenty eight Albino *Mus musculus* mice, 2 to 2.5 months old, weighed between 18 and 27 g, were used in this experiment. They were provided by the central pharmacy Constantine (Algeria). The mice were separated into four groups in four cages according to their body weight. The planned diet was given in the form of balls prepared with 0.5 mg of white flour and distilled water for 15 days. The first group (F) was fed with white flour (0.5 mg/kg/day), second group (M) was administered with with L-methionine (1 g/kg/day), third group (MP) was administerd with L-methionine and *Vitis vinifera* (1 g/kg + 500 mg/kg/day). The fourth group (P) was treated only with *Vitis vinifera* (500 mg/kg/day). Mice were housed at normal conditions of the animal house throughout the treatment period.

Blood biochemistry

At the end of experiment, mice were fasted overnight, and the blood samples were collected from the retro orbital vein into EDTA tubes by using glass capillaries. They were centrifuged immediately, and the plasma was stored at -30 °C. The values of plasma hs-CRP were measured by the immunoturbidimetric method on a Cobas integra 400 plus analyzer (Roche). Total homocysteine (t-Hcy) was estimated by competitive solid phase chemiluminescence immunoassay.

GSH glutathione assay (GSH)

We are following the same protocol mentioned by Houssem Eddine et al. (2014). After sacrificing the animals, the liver was dissected and washed with NaCl 0.9%. Then, the homogenate had been prepared with 0.5 g of the liver homogenized in 2 ml of TBS (Tris50 mM, NaCl 150 mM, pH 7.4). Next, it was centrifuged at 9000 g for 15 min at 4 °C. After that, the supernatant used for the determination of glutathione reduced (GSH), then it was measured spectrophotometrically by using 5,5′-dithiobis-(2 nitrobenzoic acid) (DTNB) as a coloring reagent, following the method of Rahman et al. (2006).

Histology

The animals were sacrificed and the aorta was removed for histological analysis. The tissues were then embedded in paraffin and cut in 5 μm thick sections and colored using heamatoxylineosin staining method.

Statistical analysis

Statistical analysis was carried out by one–way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests using statistics software package (SPSS for Windows, V. 20.0, Chicago, USA). P values < 0.05 were considered as statistically significant (common letter are not significantly different).

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Results

Phenolic composition of extract GLAV (Vitis vinifera)

The HPLC/DAD/ESI-MS analysis was used to identify all compounds directly, or by comparing the results to literature, and the analysis of leaf ingredients were permitted the identification of cyanidin-3-glucosides, -3-(6-acetyl)glucosides, and -3-(6-p-coumaroyl)glucosides (*Table 1; Fig. 1*). Nonanthocyanin phenolic compounds identified in leaf ingredients included the flavonols quercetin-3-O-galactoside, quercetin-3-O-glucuronide, quercetin-3-O-glucoside, kaempherol-3-O-galactoside, kaempherol- 3-O-glucuronide, kaempherol-3-O-glucoside, and quercetin (*Table 1; Fig. 2*).

Table 1. *Characteristics of anthocyanins (a-n) and flavonols (2-8) detected in a methanol extract of Vitis vinifera L. leaves, according to the retention time (tR), mass spectral details, UV data (λmax), corroborated by references*

Peaks	$^{x}t_R$ (min)	Compound	$[M+H]^+$ (*m/z*)	$[M-H]^-$ (*m/z*)	λmax	Fragments (MS/MS)	Reference
A	9.8	Delphinidin 3-glucoside	465.2	-	522	303	Villiers et al. (2004); Kammerer et al. (2004)
B	11.1	Cyanidin 3-glucoside	449.3	-	514	287	
C	12.6	Petunidin 3-glucoside	479.4	-	522	317	
D	13.8	Peonidin 3-glucoside	463.4	-	515	301	
E	15.0	Malvidin 3-glucoside	493.1	-	524	331	
F	18.8	Cyanidin 3-(6-*p*-acetyl) glucoside	495.4	-	519	287	Villiers et al. (2004)
G	22.3	Peonidin 3-(6-acetyl) glucoside	505.0	-	516	301	
H	23.2	Delphinidin 3-(6-*p*-coumaroyl) glucoside	611.3	-	527	303	Villiers et al. (2004); Kammerer et al. (2004)
I	25.3	Cyanidin 3-(6-*p*-coumaroyl) glucoside	595.4	-	522	287	
L	26.3	Petunidin 3-(6-*p*-coumaroyl) glucoside	625.2	-	536	317	
M	28.8	Peonidin 3-(6-*p*-coumaroyl) glucoside	609.2	-	520	301	
N	30.5	Malvidin 3-(6-*p*-coumaroyl) glucoside	639.0	-	517	331	
1	13.9	*Trans*-caftaric acid	-	311.0	320	179	Kammerer et al. (2004)
2	49.2	Quercetin 3-*0*-galactoside	-	463.1	256	301	Kammerer et al. (2004); Flamini et al. (2015)
3	50.1	Quercetin 3-*0*-glucuronide	-	479.1	256	301	
4	51.7	Quercetin 3-*0*-glucoside	-	463.1	256	301	
5	55.6	Kaempferol 3-*0*-galactoside	-	447.3	262	285	Villiers et al. (2004); Flamini et al. (2015)
6	59.0	Kaempferol 3-*0*-glucuronide	-	463.1	262	287	Flamini et al. (2015
7	61.5	Kaempferol 3-*0*-glucoside	-	477.0	262	285	Kammerer et al. (2004); Flamini et al. (2015)
8	85.5	Quercetin	-	304.0	254	273	Kammerer et al. (2004)

$^{x}t_R$Mean retention time of 3 runs

Among the extracted polyphenols from the Algerian *Vitis vinifera* L. leaves, the anthocyanins are the main chemical group, about 80.34 µg in each 1 g of freeze-dried

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sample (*Table 2*). In this extract, 12 compounds were identified by HPLC-DAD as follows: the peonidins, as the main chemical group, with about half (46.50%) of the total anthocynins with 37.35 µg/g freeze-dried sample in each g of the predominant compound was peonidin 3-glucoside, the second abundant group was the cyanidins, with cyanidin-3-glucoside, of 78% per the total of cyanidins tailed by cyanidin-3-(6-acetyl)-glucoside (13%) and cyanidin-3-(6-*p*-coumaroyl)-glucoside (9%). The malvidins represented the third abundant group with 12.98 µg/g of sample and 2 compounds (malvidin 3-glucoside and malvidin 3-(6-*p*-coumaroyl) glucoside), the glucoside forms were the most abundant; the two remaining chemical classes, delphinidins and petunidins were contributed to total anthocyanins for 6.30% and 2.63%, respectively. Concerning the non-anthocyanins it is interesting to note the high concentrations of trans-caftaric acid and quercetin 3-*0*-glucuronide. Quercetins were the most abundant flavonols and were represented by quercetin (5.2%) and the glucuronide (76.5%), galactoside (8.3%) and glucoside (10%) forms. Kaempferols were presented 11.40% of flavons and the most abundant form was the galatoside (82.2%) one. For these compounds it is interesting to note the very high concentration of the glucuronide form and the notable concentration of quercetin.

Table 2. *Phenolic composition of Vitis vinifera L. leaves extract according to HPLC-DAD chromatography*

Anthocyanins[z]					
Cyanidins	**Delphinidins**	**Petunidins**	**Peonidins**	**Malvidins**	**Total anthocyanins**
22.83 µg 28.42%[y]	5.06 µg 6.30%	2.12 µg 2.63%	37.35 µg 46.50%	12.98 µg 16.15%	80.34 µg
Flavonols[z]					
Quercetins		**Kaempherols**		**Total flavanols**	
39.70 µg 88.60%[w]		5.11 µg 11.40%		44.81 µg	
Cinnamates					
***Trans*-caftaric acid**					
9.52 µg					

[x]Expressed as µg malvidin-3-glucoside equivalents/g freeze-dried leaves
[y]% of total anthocyanins: Σ Cyandins = cyanidin-3-glucoside + cyanidin-3-(6-acetyl)-glucoside + cyanidin-3-(6-*p*-coumaroyl)-glucoside; Σdelphinidins = delphinidin-3-glucoside + delphinidin-3-(6-*p*-coumaroyl)-glucoside; Σ petunidins = petunidin-3-glucoside + petunidin 3-(6- *p*-coumaroyl)-glucoside; Σ peonidins = peonidin-3-(6-acetyl)-glucoside + peonidin-3-(6-*p*-coumaroyl)-glucoside; Σ malvidins = malvidin-3-glucoside + malvidin-3-(6-*p*-coumaroyl)-glucoside
[z]Expressed ad µg quercetin or keampherol equivalents/g freeze-dried leaves
[w]% of total flavonols: Σ Quercetins = quercetin-3-*O*-galactoside + quercetin-3-*O*-glucuronide + quercetin-3-*O*-glucoside + quercetin; Σ kaempherols = kaempherol-3-*O*-galactoside + kaempherol-3-*O*-glucuronide + kaempherol-3-*O*-glucoside
The anthocyanin/flavonol ratio = 1.79

Effect of Vitis vinifera extract on homocysteine induced inflammatory endothelial damage in cardio vascular diseases.

In this study, we have taken only 3 values from each group because some are outliers.

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As shown in *Figure 3*, the total Hcy levels in groups F (7.56 ± 0.21 µmol/l), M(12.20 ± 0.78 µmol/l), MP(9.06 ± 0.34 µmol/l) and P(7.24 ± 0.40 µmol/l) were showed a significant difference between groups in mice during 15 days of treatment $P \leq 0.05$. The Tukey test was showed that the homocysteine concentration in mice administered with L-methionine was increased highly significantly in group M when it was compared to the control group $P \leq 0.05$. However the homocysteine concentration was decreased significantly in the group of mice administered with L-methionine and treated with *Vitis vinifera L* when it was compared to the group (M) $P \leq 0.05$. Exceptionally, no significant changes were observed between the group treated only by *Vitis vinifera* leaves and the control group ($P > 0.05$).

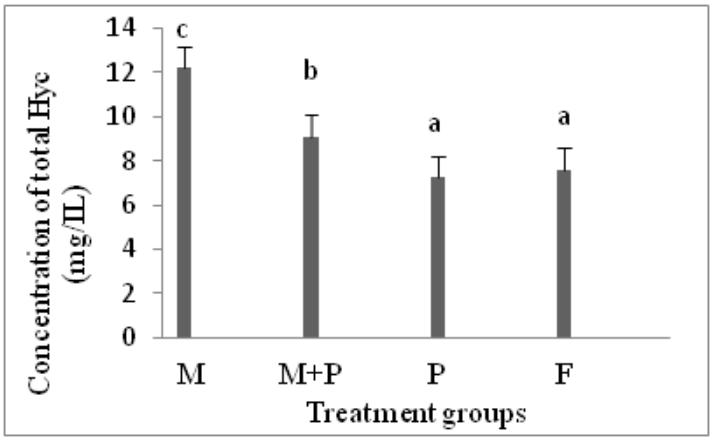

Figure 3. *Total Hcy level in the groups treated during 15 days. Values are the means ± SEM (n)*

The concentrations of hs-CRP in groups F (0.14 ± 0.017 mg/l), M (0, 28 ± 0,012 mg/l), MP (0.18 ± mg/l) and P (0.14 ± 0,012) were showed a significant difference between groups $P \leq 0.05$ (*Fig. 4*). The Tukey test was revealed that the hs-CRP concentration in the group (M) was increased highly significantly when it was compared to the groups (F) and (P) $P \leq 0.01$. However the concentration of hs-CRP was decreased significantly in the group (MP) when it is compared to the group (M).

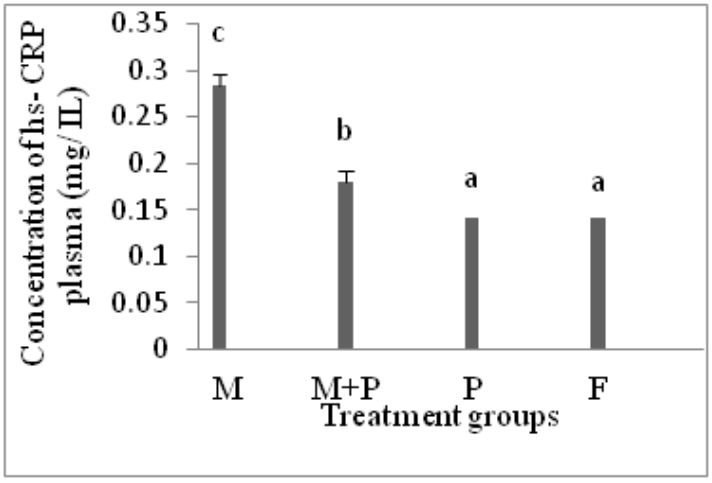

Figure 4. *Concentration of hs-CRP plasma in the groups treated during 15 days. Values are the means ± SEM (n)*

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Results demonstrated that the concentration of the glutathione reduced in groups F(29.28 ± 1.48 nmol/mg), M (19.38 ± 1.21 nmol/mg), MP(26.74 ± 1.06 nmol/mg) and group P(30.17 ± 1.37 were showed a significant difference between groups $P \leq 0.05$ (*Fig. 5*). The Tukey test showed that the concentration of the glutathione reduced in group M was decreased highly significantly when it is compared to the control group $P \leq 0.01$. However the concentration of GSH was increased in the groups treated with *Vitis vinifera L.*

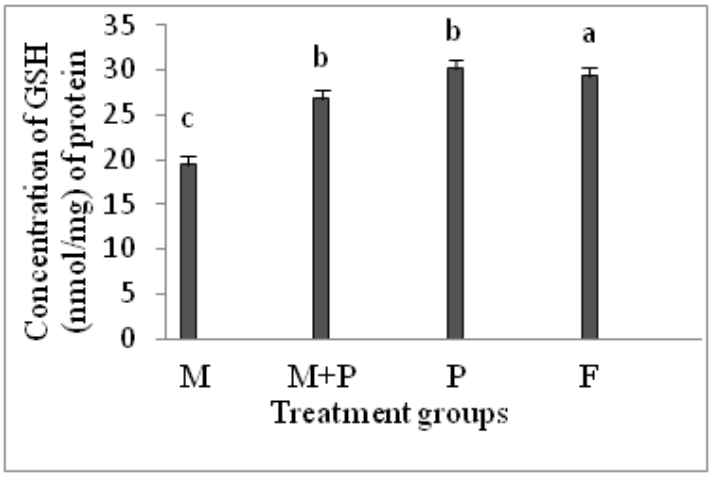

Figure 5. *Concentration of GSH in the groups treated during 15 days. Values are the means ± SEM (n)*

Aorta histological

The results of the histological investigation showed a clear modification in the aorta. The group (M) which was fed with 1 g/kg of L-methionine appeared with oval nuclei of muscular fiber, desquamation of endothelial cells. However, in the control group (F), the aortic sections were showed an intact endothelium. The group (MP) which was fed with leaves extracts of the *Vitis vinifera* and 1 g/kg of L-methionine was showed only slight modifications including some oval nuclei of muscular cells (*Figs. 6*, *7* and *8*).

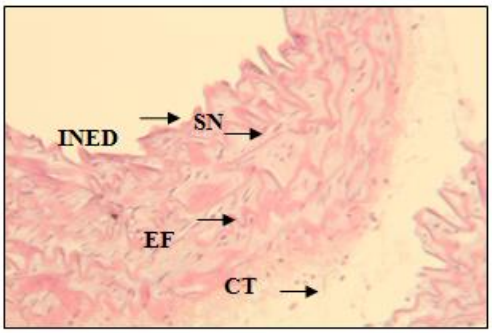

Figure 6. *Longitudinal section of arch aorta 15 days of flour application. Heamatoxylin Eosin staining (x100). EF: elastic fiber, SN: spindle nuclei, CT: connective tissue, INED: intact endothelium*

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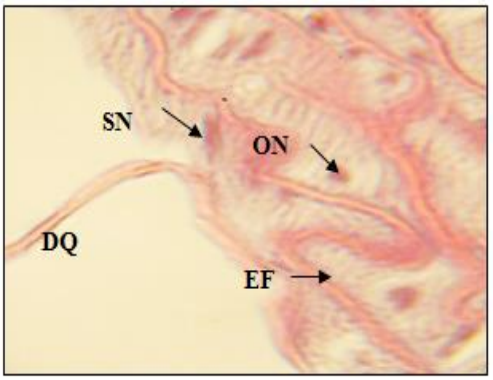

Figure 7. *Longitudinal section of arch aorta 15 days of L-methionine application. Heamatoxylin Eosin staining (x400). EF: elastic fiber, DQ: desquamation, ON: oval nuclei, SN: spindle nuclei*

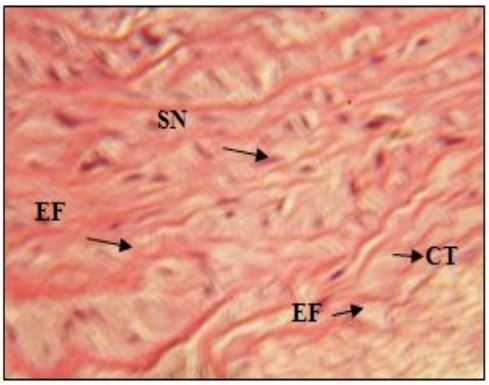

Figure 8. *Longitudinal section of arch aorta 15 daysof L-methionine and Vitis vinifera leaves application.Heamatoxylin Eosin staining (x100).EF: Elastic Fiber SN: Spindle nuclei CT: connective tissue*

Discussion

In this study, the High-performance liquid chromatography (HPLC) was used for the separation and quantification of polyphenols in leaves of *Vitis vinifera. L* species, the Algerian variety. The results showed that high levels of phenols; anthocyanins, flavonols and trans-caftaric acid. The results is agrees with the work of (Monagas et al., 2006) who reported that HPLC-DAD/ESI-MS analysis of *V. Vinifera* spp. leaves ingredient allowed the identification of anthocyanidin-3-glucosides,-3-(6-acetyl)glucosides, and -3-(6-p-coumaroyl) glucosides and the flavonols quercetin-3-O-glucoronide, quercetin-3-O-glucuronide, quercetin-3-O-glucoside, kaempherol-3-O-galactoside, kaempherol-3-O-glucuronide, kaempherol-3-O-glucoside, and quercetin like non-anthocyanin content. The chromatograph pattern and the compounds identified agree with other paper on the same subject (Monagas et al., 2006). Trans-Caffeoyltartaric acid (trans-caftaric acid) was the only hydroxycinnamic acid derivative identified in the studied leaf ingredients and results obtained were in agreement also with (Monagas et al., 2006). According of the results obtained, GLAV contain much higher concentration of peonidins, Trans-Caffeoyltartaric acid (trans-caftaric acid) and

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Quercetins but the concentration of Kaempherols is found inferior than values mentioned by the results of Monagas et al. (2006). The differences observed between the results may be attributed to the period of the plant growth cycle, variety, cultivar conditions, weather and finally to the processing and preparation because leaves used by Monagas et al. (2006) were cultivated using the commercial dietary technology.

Homocystein (Hyc) is considered as a risk marker and can be used for screening patients of high menace for cardiovascular events (Refsum et al., 1998). In this study, the oral administration of high dose of L-methionine 1 g/kg/day during 15 days showed a significant increase in the level of plasma tHyc compared to the control group. These results are in agreement with those found by (Zerizer, 2006), who showed that the high oral methionine load is the direct cause of the elevation of the total homocysteine (tHcy), the sum of all homocysteine forms that exist in plasma or serum, therefore having hyperhomocysteinemia means the elevation of total homocysteine (Bernardo et al., 2004). Two hypotheses were formulated to explain the atherogenicity of hyperhomocysteinemia. The lipid hypothesis stipulates that the alteration of lipoprotein metabolism secondarily induces an involvement of the vascular wall, and the inflammatory hypothesis is dominated by the direct aggression of the cells and vascular connective tissue (Demuth et al., 2000).

The levels of hs-CRP and GSH have been exploited to monitor the effects of GLAV on the inflammatory and oxidative effect caused by hyperhomocystemia.

Currently and in several scientific researches, the CRP is used as a marker of cardiovascular risk (Folsom et al., 2002). The group (M) showed significant high level of hs-CRP compared with the control one. Benmbarek et al. (2013) confirmed that methionine at dose of 200 mg/kg/day administered to mice, during the 21 days period, increased significantly the levels of plasma hs-CRP. This result was considered as an initiative of the inflammatory process, which was confirmed by the histological investigation of the aorta. Results are in agreement with the previous experimental studies of Benmbarek et al. (2013) who found that Hyperhomocysteinemia as angiotoxic and toxic activity explained by the loss and degeneration of the endothelium, formation of foam cells in the different sections of the aorta, change in the smooth muscle cells nuclei forms from a fusiform aspect to a rounded appearance, and the alteration of the cardiac muscle and liver necrosis. In addition, Zerizer and Naimi (2004) reported the structural alterations in the aorta, heart and liver caused by the administration of high doses of methionine.

Substantially reduced (GSH) involved in maintaining the redox potential of the cell cytoplasm and in a number of detoxification reactions and scavenging reactive oxygen species (Haleng et al., 2007). Result demonstrated that the group (M) showed a significant decrease in the level of GSH compared to the control group (F). These results confirmed an oxidative stress generated by the reactive sulfhydryl group (-SH) in the homocysteine (Jacobsen, 2000), which is quickly oxidized, leading to the formation of Hcy, mixed disulfides and Homocysteinethiolactone. The oxidation of the -SH group generates superoxide anion O2-, hydrogen peroxide H_2O_2 and hydroxyl radicals OH- (Zitoun, 1998). Zeng et al. (2004) confirmed that the Hcy induced the production of MCP1 and IL-8. Additionally, a recent study suggested that Hcy induced the production of O2- in vascular smooth muscle cells (Wang et al., 2001). Yalçinkaya et al. (2009) demonstrated that a high methionine diet induced oxidative stress in serum, heart, and aorta in rabbits.

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The group (MP) treated by leaf of *Vitis vinifera* and L-methionine (500 mg/kg and 1 g/kg) noted a significant decrease in the levels of tHyc compared to group (M). The same group rectified significantly the level of hs CRP compared with the control group and group (M). At the same time, the group (MP) re-established significantly the level of GSH compared to the group (M).

These significant relationships between the parameters can be explained as the effect of the existed phenols in the GLAV. Exactly, the group (MP) treated by the GLAV was able to restore the level of the hs- CRP and GSH and maintain the correlation between the three parameters and could correct the damaged cells in aorta.

The results confirmed that GLAV (500 mg/kg/day) has an antioxidant and anti-inflammatory effects induced by Hyperhomocysteinemia in mice fed by a high dose of L-methionine 1 g/1 kg/day during 15 days. Benmbarek et al. (2013) asserted that S. mialhesi extract lowered the plasma hs-CRP and corrected the damaged cells.

In addition, the phenols, which are ubiquitous in almost all plant foods could decrease the risk of the occurrence of considerable number of diseases, particularly those related to aging and oxidative injury (cancer, cardiovascular diseases and neurodegenerative) (Hennebelle et al., 2014). While, low circulating levels of polyphenols (maximum, few μmol/L) compared to those of other endogenous antioxidants (GSH and acid uric) or exogenous (vit E as well as vit C) do not allow to envisage a direct antioxidant action of polyphenols in the body. This, however, with the exception of the gastrointestinal tract where the polyphenols present in large quantities can act as scavengers of free radicals. Today at the level of organism, polyphenols are perceived as molecules "Signal" (Mornad et Milenkovic, 2014), that could stimulate multi-target modes of action. Many in-vitro studies showed that flavonoids could affect their biological targets by modulating some enzymatic activities, gene expression or cell signaling, interacting with membrane or cell receptors, or via epigenetic regulations (Farga et al., 2010). The diversity of these potential mechanisms of action explains the broad spectrum of activities flavonoids observed *in-vivo*, including anti-inflammatory activities, antioxidant, anti-angiogenic, anti-proliferative or pseudo-estrogenic (Mornad and Milenkovic, 2014).

Conclusion

The grape leaves have a good antioxidant and anti-inflammatory activities. Where, it could be directly related to the high content of active compounds like peonidins, Trans-Caffeoyl tartaric acid (trans-caftaric acid) and Quercetins. More studies in this area are required further to find new ways and new efficient molecules from *Vitis vinifera* leaves to treat degenerative diseases and to slow the aging process induced by oxidative intermediate products and other pro-inflammatory components.

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Paper 02

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Article

Total Phenols from Grape Leaves Counteract Cell Proliferation and Modulate Apoptosis-Related Gene Expression in MCF-7 and HepG2 Human Cancer Cell Lines

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Abstract: Grape leaves influence several biological activities in the cardiovascular system, acting as antioxidants. In this study, we aimed at evaluating the effect of ethanolic and water extracts from grape leaves grown in Algeria, obtained by accelerator solvent extraction (ASE), on cell proliferation. The amount of total phenols was determined using the modified Folin-Ciocalteu method, antioxidant activities were evaluated by the 2,2-diphenyl-l-picrylhydrazyl free radical (DPPH*) method and ˙OH radical scavenging using electron paramagnetic resonance (EPR) spectroscopy methods. Cell proliferation of HepG2 hepatocarcinoma, MCF-7 human breast cancer cells and vein human umbilical (HUVEC) cells, as control for normal cell growth, was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay (MTT). Apoptosis-related genes were determined by measuring Bax and Bcl-2 mRNA expression levels. Accelerator solvent extractor yield did not show significant difference between the two solvents (ethanol and water) ($p > 0.05$). Total phenolic content of water and ethanolic extracts was 55.41 ± 0.11 and 155.73 ± 1.20 mg of gallic acid equivalents/g of dry weight, respectively. Ethanolic extracts showed larger amounts of total phenols as compared to water extracts and interesting antioxidant activity. HepG2 and MCF-7 cell proliferation decreased with increasing concentration of extracts (0.5, 1, and 2 mg/mL) added to the culture during a period of 1–72 h. In addition, the expression of the pro-apoptotic gene Bax was increased and that of the anti-apoptotic gene Bcl-2 was decreased in a dose-dependent manner, when both MCF-7 and HepG2 cells were cultured with one of the two extracts for 72 h. None of the extracts elicited toxic effects on vein umbilical HUVEC cells, highlighting the high specificity of the antiproliferative effect, targeting only cancer cells. Finally, our results suggested that ASE crude extract from grape leaves represents a source of bioactive compounds such

as phenols, with potential antioxidants activity, disclosing a novel antiproliferative effect affecting only HepG2 and MCF-7 tumor cells.

Keywords: grape leaves; ASE; TP; Antioxidant activities; Antiproliferative; pro-apoptotic effects; Gene expression; Nutraceuticals

1. Introduction

Oxidative stress is a pathogenetic mechanism associated with several diseases, including atherosclerosis, neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, cancer, diabetes mellitus, inflammatory diseases, as well as psychological diseases or aging processes [1]. Indeed, increased formation of free radicals (FR) can promote the development of malignancy, and "normal" rates of FR generation may account for the increased risk of cancer development in the elderly [2]. Cancer is the major cause of morbidity and mortality in modern society. The number of deaths by cancer in 2008 was estimated to be 7.6 million, a number predicted to double by 2030 [3]. In developed countries, cancer is the main cause of death after cardiac disease [4]. Many treatments against cancer are possible, such as surgical removal, chemotherapy, radiation therapy and immunotherapy.

Apoptosis, or programmed cell death, is a normal and fundamental event that occurs in a highly regulated and precise manner. This process plays a key role in normal tissue development and maturation, maintaining the homeostasis in the body by controlling the immune system. Apoptosis is the most potent defense against cancer since it is the mechanism used by metazoans to eliminate deleterious cells. Furthermore, a large number of chemo preventive agents exert their effectiveness by inducing apoptosis in transformed cells, as shown both in vitro and in vivo [5,6]. Since apoptosis provides a physiologic mechanism to eliminate abnormal cells, dietary factors affecting apoptosis can elicit an important effect on carcinogenesis. For these reasons, activation of apoptosis by dietary factors in pre-cancerous cells may represent a preventive mechanism (chemoprevention) [6,7].

Nearly 90 out of 121 drugs prescribed to treat cancer originate from plants [8]. The term "nutraceutical" was coined in 1989 by Stephen De Felice to define "food, or parts of a food, that provide medical or health benefits, including the prevention and treatment of disease" [9–11]. Many studies demonstrate that grapes are rich in anthocyanins, flavanols, flavonoids, terpenes, organic acids, vitamins, carbohydrates, lipids and enzymes [12,13]. These findings have created considerable interest in grape leaves as a promising source of compounds with nutritional properties and biological potential. Moreover, the use of grape leaves provides a way of solving the disposal problems arising from the large amounts of industrial residues generated by the wine and juice industries [14,15].

Extraction is the most important step to recover and isolate bioactive molecules from plant materials. Various extraction techniques have been developed to obtain nutraceuticals from plants in order to shorten extraction time, reduce solvent consumption, increase extraction yield, improve the quality of extracts and increase pollution prevention [16]. Among those, accelerated solvent extraction (ASE) is a solid-liquid extraction process performed at elevated temperature and under pressure to maintain the solvent in its liquid state. The solvent remains below its critical condition during ASE. The increased temperature accelerates the extraction kinetics and the elevated pressure keeps the solvent in the liquid state, thus achieving a safe and rapid extraction. The only disadvantage of ASE is the high cost of the needed equipment [17].

The aim of the present study was to analyze the polyphenol anti-oxidative and anti-proliferative properties of water and ethanol ASE crude extracts from grape leaves grown in Medea (Algeria).

2. Results

2.1. Yield and Total Phenolic Content

Table 1 shows the yield and total phenolic content of ethanolic and aqueous ASE crude extracts obtained from grape leaves. The aqueous extract gave a higher total phenolic yield (22.8 ± 3.21%) as compared to ethanol (18.87 ± 0.6%), despite not being statistically significant ($p = 0.116$). However, the ethanolic extracts exhibited larger amounts of TP (around 2.8 times) as compared to the water extract ($p = 0.001$). The ethanol polarity might be responsible for the observed TP content difference.

Table 1. Yield extraction (%), total phenols and EPR-spin trapping and DPPH-radical scavenging activity (IC50) of ethanolic and water crude extracts obtained by accelerator solvent extraction (ASE).

Type of Extracts	Total Phenols (mg GAE/gr DW ± SD) [y]	Yield (% ± SD)	IC50·OH (mg/mL ± SD)	IC50 DPPH (mg/mL ± SD)
WACE	55.41 ± 0.11 [a]	22.8 ± 3.21 [a]	0.67 ± 0.53 [a] $R^2 = 0.9791$	0.15 ± 0.41 [a] $R^2 = 0.9711$
EACE	155.73 ± 1.20 [b]	18.87 ± 0.6 [a]	0.64 ± 0.71 [a] $R^2 = 0.9989$	0.09 ± 0.32 [b] $R^2 = 09922$

[y] GAE: gallic acid equivalent; DW: Dry weight; SD: standard deviation; IC50: sample concentration at which 50% of the free radical activity was inhibited. a: ASE water crude extract; b: ASE ethanolic crude extract. The unlike letters represent values significantly different at $p < 0.05$

2.2. DPPH and EPR Radical-Scavenging Activity

The antioxidant capability was expressed as the quantity of antioxidant inducing a 50% decrease in DPPH concentration or a 50% inhibition of the hydroxyl radical production (IC50). The quenching efficiency of DPPH or hydroxyl radical is inversely proportional to the IC50. Table 2 shows the IC50 of grape leaves ethanolic and aqueous crude extracts. The ethanolic extract of grape leaves showed higher activity of the scavenging DPPH radical (0.09 mg/mL) as compared to the aqueous extract (0.15 mg/mL) ($p = 0.035$). Ethanolic and water extracts provided IC50 of 0.67 (±0.53) and 0.64 (±0.71) mg/mL respectively. The trapping of hydroxyl radical did not show any significant difference between the two extracts ($p = 0.181$).

Table 2. IC50* of grape leaves ethanolic and water ASE crude extracts on MCF-7, HepG2 and HUVEC cells.

Extract / Cells	MCF-7	HepG2	HUVEC
WACE [y] IC50* (mg/mL)	0.71	1.1	>>2
EACE [x] IC50* (mg/mL)	0.43	0.7	>>2

* IC50: sample concentration at which 50% of cell proliferation was inhibited; [y] WACE: ASE aqueous crude extract; [x] EACE: ASE ethanolic crude extract.

2.3. Effect of Grape Leaves EACE and WACE Extract on HUVEC Cell Proliferation

Both the ethanolic (EACE) and aqueous (WACE) extracts were not toxic for HUVEC cells, with the IC50 being higher that 2 mg/mL. Ethanolic and water extracts inhibited HUVEC cells proliferation in a dose-dependent manner ($p = 0.01$ and HUVEC cells induced an inhibition of cell growth (96%) at 10 μM (Figure 1)).

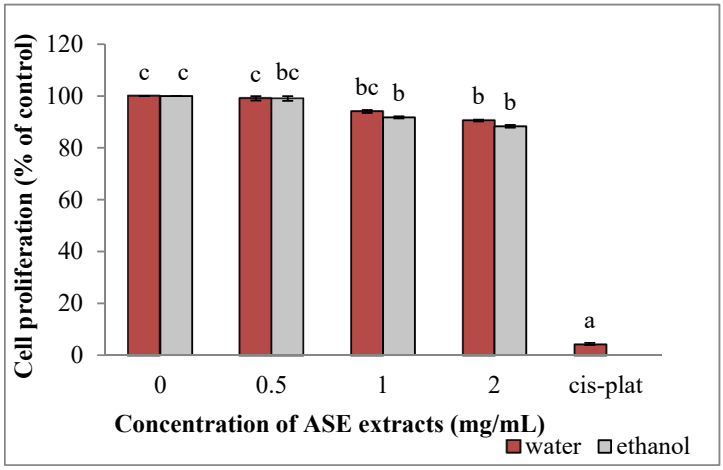

Figure 1. Effect of ASE crude extracts on HUVEC cell proliferation (untreated group: concentration = 0). Data are expressed as mean ± SD, n = 3. Bars marked by unlike letters within a group are significantly different at $p < 0.05$, according to Duncan's Multiple Range Test (DMRT).

2.4. EACE and WACE Extract Counteract HepG2 Proliferation

The survival of HepG2 cells was significantly reduced following incubation with ethanol ($p = 0.001$) and water extracts ($p = 0.001$) (cell proliferation is expressed as the mean percentages of viable cells relative to untreated cells) (Figure 2). In addition, inhibition of HepG2 cell proliferation by both extracts were dose-dependent. In particular, IC50 was obtained when 0.7 mg/mL or 1.1 mg/mL of ethanolic or water extracts, respectively, were added to the culture medium. In all cases, ethanolic extracts were significantly more active than water extracts ($p = 0.001$). The maximum growth inhibition was obtained using Cisplatin (93.52%), representing the positive control, followed by 2 mg/mL ethanolic extracts (82.5%) and 2 mg/mL water extracts (68.63%).

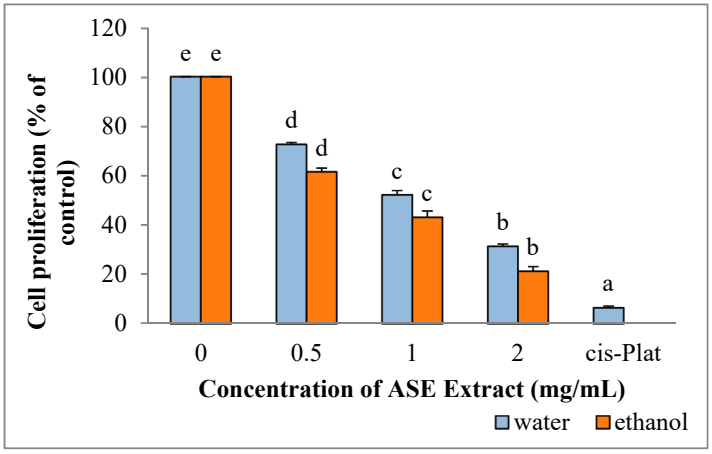

Figure 2. Effect of ASE crude extracts on of HepG2 cell proliferation. Each value is expressed as mean ± SD, n = 3 (untreated group: concentration = 0). Bars marked by unlike letters within a group are significantly different at $p < 0.05$, according to Duncan's Multiple Range Test (DMRT).

2.5. EACE and WACE Extracts Influence the Expression of Apoptosis-Related Genes in HepG2 Cells

HepG2 cultured in the presence of EACE or WACE exhibited a significant increase in Bax mRNA levels in a concentration-dependent manner, as compared to untreated control ($p < 0.05$) (Figure 3). Moreover, Bcl-2 gene expression was down-regulated in a concentration-dependent manner ($p < 0.05$) (Figure 4). The effect of ethanolic extracts was more prominent on HepG2 cells than water extracts ($p = 0.002$). In particular, the maximum effect on both Bax and Bcl-2 genes was observed using the highest concentration (2 mg/mL) of ethanolic extracts.

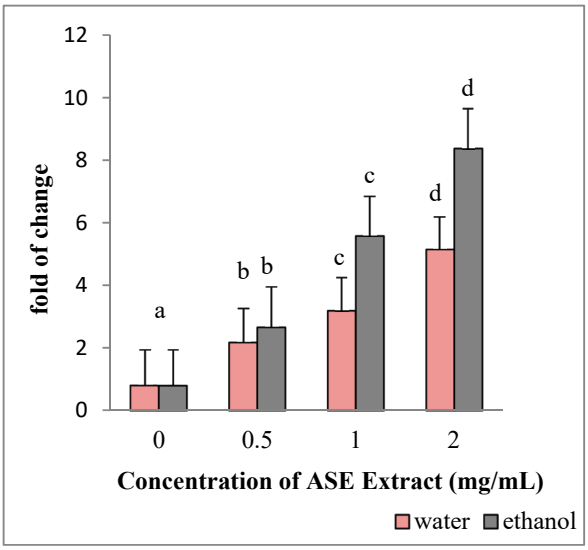

Figure 3. Effect of ASE crude extracts on Bax gene expression in HepG2 cells. The mRNA levels for each gene are expressed as fold of change ($2^{-\Delta\Delta Ct}$) relative to the untreated control (defined as 1) (mean ± SD; n = 3) and normalized to the Glyceraldehyde-3-Phosphate-Dehidrogenase (GAPDH). Data are expressed as mean± SD referred to the control. Bars marked by unlike letters within a group are significantly different at $p < 0.05$, according to Duncan's Multiple Range Test (DMRT).

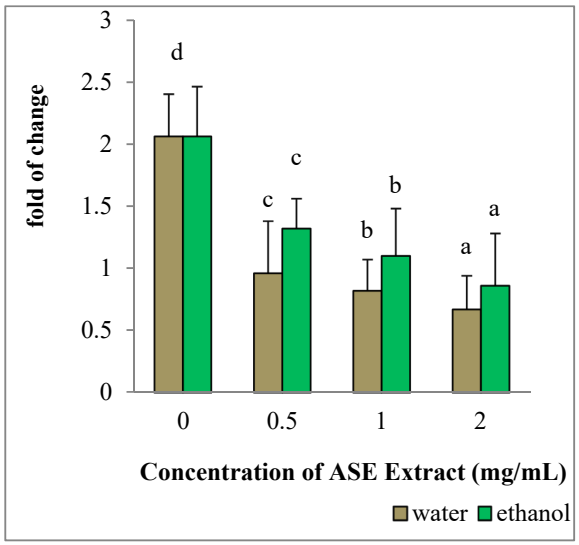

Figure 4. Effect of ASE crude extracts on Bcl-2 gene expression in HepG2 cells. The mRNA levels for each gene are expressed as fold of change ($2^{-\Delta\Delta Ct}$) relative to the untreated control (CTRL-), defined as 1 (mean ± SD; n = 3), and normalized to the Glyceraldehyde-3-Phosphate-Dehidrogenase (GAPDH). Data are expressed as mean ± SD referred to the control. Bars marked by unlike letters within a group are significantly different at $p < 0.05$, according to Duncan's Multiple Range Test (DMRT).

2.6. EACE and WACE Extracts Influence MCF-7 Proliferation

Similar to what was observed in HepG2 cells, both crude extracts significantly inhibited MCF-7 cell proliferation (Figure 5). In particular, the IC50 for EACE and WACE of grape leaves was 0.43 mg/mL and 0.71 mg/mL, respectively. The ethanolic extracts were significantly more active than the water extracts ($p = 0.002$). The largest percentage of growth inhibition was obtained by Cisplatin (99.34%), followed by ethanolic (88.56%) and water extracts (79.31%) (Figure 5). Results revealed that MCF-7 cells were more sensitive to extracts than HepG2 cells.

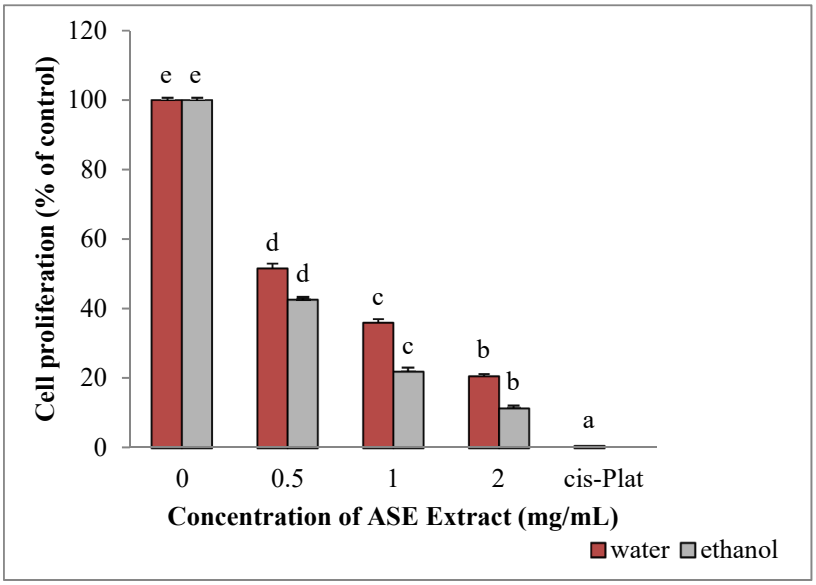

Figure 5. Effects of ASE crude extracts on MCF-7 cell proliferation (concentration 0 corresponding to the untreated group). Data are expressed as mean ± SD, n = 3. Bars marked by unlike letters within a group are significantly different at $p < 0.05$, according to Duncan's Multiple Range Test (DMRT).

2.7. EACE and WACE Extracts Influenced the Expression Of Apoptosis-Related Genes in MCF-7 Cells

Ethanol and water extracts significantly modulated Bax and Bcl-2 mRNA expression levels in MCF-7 cells in a concentration-dependent manner, with Bax expression being significantly upregulated ($p = 0.001$) and Bcl-2 significantly down-regulated ($p = 0.002$). The maximum effect was observed at the highest concentration (2 mg/mL) of ethanolic or water extracts (Figures 6 and 7).

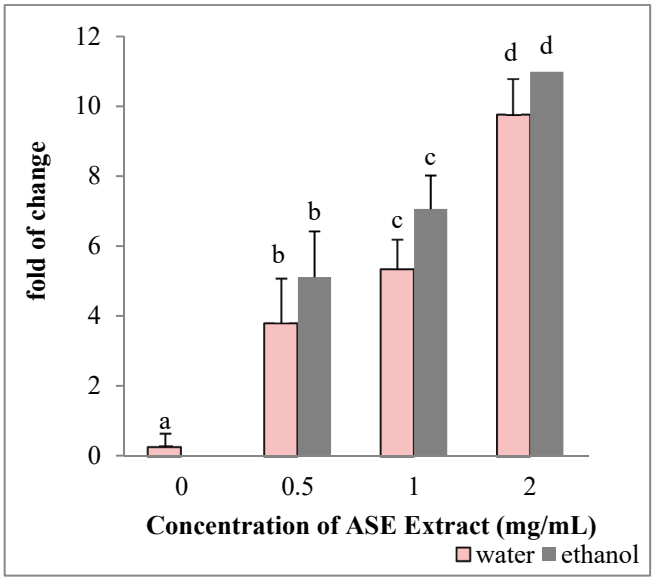

Figure 6. Effect of ASE crude extracts on Bax gene expression in MCF-7 cells. The mRNA levels are expressed as fold of change ($2^{-\Delta\Delta Ct}$) as compared to untreated HepG2 cells (defined as 1) (mean ± SD; n = 3) and normalized to GAPDH. Bars marked by unlike letters within a group are significantly different at $p < 0.05$, according to Duncan's Multiple Range Test (DMRT).

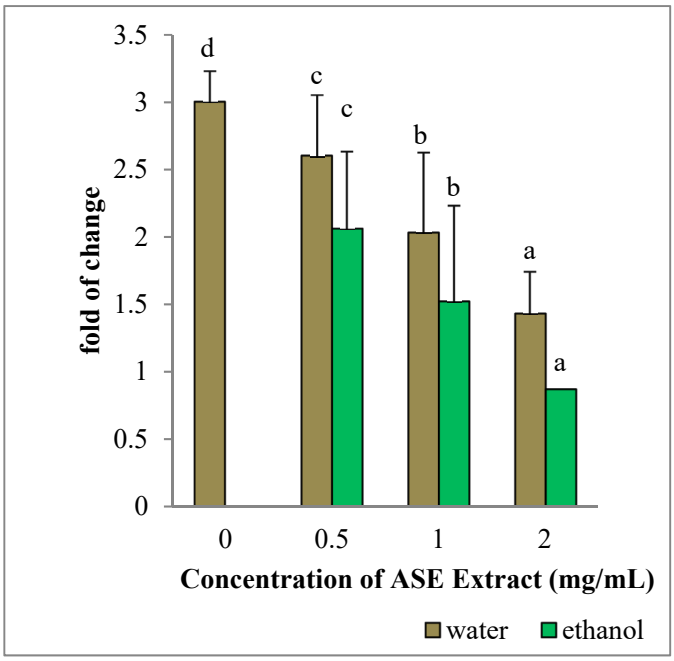

Figure 7. Effect of ASE crude extracts on Bcl-2 gene expression in MCF-7 cells. The mRNA levels are expressed as fold of change ($2^{-\Delta\Delta Ct}$) as compared to untreated HepG2 cells (defined as 1) (mean ± SD; n = 6) and normalized to GAPDH. Bars marked by unlike letters within a group are significantly different at $p < 0.05$, according to Duncan's Multiple Range Test (DMRT).

3. Discussion

Plant bioactive compounds have drawn increasing attention due to their potent antioxidant properties and their marked effects in the prevention of various oxidative-stress-associated diseases, such as cancer. In the last few years, the identification and development of these compounds or extracts from different plants has become a major area of health- and medical-related research [18]. Phenolic compounds are considered as bioactive compounds, widely present in all parts of plant and crude extracts [19].

In this study, we utilized the accelerator solvent extraction method to prepare crude extracts of grape leaves, grown in Algeria, in ultrapure water and 60% ethanol. We aimed at evaluating the anti-proliferative effects of these extracts on HepG2 hepatocarcinoma cells and MCF-7 breast cancer cells. The amount of total phenols and the antioxidant activity were evaluated by scavenging DPPH* and trapping of hydroxyl radical using EPR-spin trapping technique. Then, cell viability was analyzed by using different concentrations of the extracts.

This study showed for the first time, the extraction of bioactive compounds such as phenolic compounds from grape leaves by ASE. ASE provided fast (10 min), easy (automated technique), safe (no direct exposure to the solvent) and inexpensive (in 34 mL of solvent) extraction, leading to high yields and high phenolic contents. Leelavinothan and Arumugam (2008) found that grape leaves contain 99 mg of gallic acid equivalents (mg GAE)/g of phenolic compounds in 70% hydroalcoholic solvent after 72 h of extraction [20], a value lower than the one obtained with the extraction methods described in the present study and requiring a longer extraction time and more solvent. Orhan et al., (2007) describe a phenolic compound yield of 16,07% by extracting 500 g of *Vitis vinifera* dried powder leaves with 80% ethanol at room temperature (5 L * 6 times) [21]. The pressure exerted by ASE allows the extraction cell to be filled faster and helps to force liquid into the solid matrix. Elevated temperatures enhance the diffusivity of the solvent, resulting in an increased extraction kinetic [22–24]. Consequently, ASE may be used to obtain a higher yield in an extremely short time as compared to all previously described methods. Indeed, in recent years, ASE has been successfully applied to the

extraction of phenolic compounds from different plant materials, such as grape seeds and skin [25–27] apples [28], spinach [29], eggplants [30] and barley flours [31].

Electron paramagnetic resonance (EPR) spin trapping has become an indispensable tool for the specific detection of reactive oxygen free radicals in biological systems [32]. The EPR spin-trapping technique was used to study the ability of ASE grape leaves extracts to quench OH radicals, which are common reactive oxygen species associated with oxidative cell damage [33]. The hydroxyl radical reacts unselectively and very quickly with any chemical compound able to lose a hydrogen atom [34]. Our results indicate that water and ethanol grape leaf extracts possessed similar ˙OH radicals quenching activity. In water extract, the content of TP, despite being lower than that of ethanol, was high enough to react with the hydroxyl radicals produced, thus excluding any dose-dependent mechanism in the reaction between antioxidants and ˙OH.

DPPH* free radical was used to evaluate the ability of phenolic compounds to transfer labile hydrogen atoms to radicals [32]. Our extracts showed high capability to scavenge DPPH*, due to the presence of different polyphenols, including flavonoids, which can be found in grape leaves [35,36]. Generally, the chemical structure of flavan-3-ol family grants a good antioxidant response towards DPPH. The hydrogen-donating substituents (hydroxyl groups), attached to the aromatic ring structures of flavonoids, allow for a redox reaction able to scavenge free radicals [21,37].

Apoptosis can be activated through two major pathways, the mitochondria-dependent pathway and the death-receptor-dependent pathway. In the mitochondria-dependent signaling pathway, the Bcl-2 family of proteins is divided into two groups: suppressors of apoptosis (e.g., Bcl-2, Bcl-XL, Mcl-1) and activators of apoptosis (e.g., Bax, Bok, Hrk, Bad). The Bax/Bcl-2 ratio might represent a critical factor influencing cell behavior. Suppression of Bcl-2 promotes apoptosis in response to several stimuli, including anticancer drugs [38]. Bax is a pro-apoptotic protein residing in the cytosol in an inactive form and translocating, after activation, to the mitochondria, where it plays an important role in mitochondria-mediated apoptosis. Activated Bax, either in homo-oligomeric form or as complex with other proteins, creates pores in the outer mitochondrial membrane, which leads to the leakage of ions, essential metabolites and cytochrome c from mitochondria to cytosol, thus promoting cell death [39]. Our results demonstrated that grape leaves have an anti-proliferative effect on HepG2 and MCF-7 cells. EACE and WACE markedly inhibited HepG2 and MCF-7 cell viability.

In cells cultured with these extracts, the mRNA levels of the anti-apoptotic factor, Bcl-2, were downregulated, while the expression of the pro-apoptotic gene Bax, was significantly induced. Within this context, other authors have demonstrated that molecules as Diazaphenothiaznes exert an antiproliferative activity in MCF7 cells and C32 human amelanotic melanoma, by regulating BAX and BCL2 gene expression [40,41].

Deepak et al. (2015) show that desert plant extracts are able to induce apoptosis in HepG2 cells. They also describe an upregulation of Bax, Bad, cytochrome c, caspase-3, caspase-7, caspase-9 and poly (adenosine diphosphate-ribose) polymerase [42]. Furthermore, the *Allium atroviolaceum* flower extracts was found to inhibit HepG2 cell growth, revealing a sub-G_0 cell cycle arrest, changes in morphological features and annexin-V and propidium iodide positive staining, which correlates with Bcl-2 down-regulation and caspase-3 activity [43]. Lu Y et al. (2011) report that injectable seed extracts from *Coix lacryma-jobi* L. induce apoptosis in HepG2 cells, with elevated and prolonged expression of caspase-8, which do not significantly influence the expression of Bcl-2 [44]. Moreover, Jun et al. (2009) report that quercetin can inhibit proliferation and induce apoptosis in HepG2 cells by decreasing the levels of surviving cells and Bcl-2 protein expression, and significantly increasing the protein levels of p53 [38].

We found that the ethanolic crude extracts were able to induce a larger anti-proliferative effect as compared to the aqueous crude extracts, which may be due to the different amount of phenols detected in the two different extracts. Nevertheless, further experiments are needed in order to understand if apoptosis could definitely explain the antiproliferative effects induced by the extracts tested in the present study.

Our extracts showed growth inhibition in MCF-7cells, confirming what has been previously described by other authors using different plant extracts. Blassan et al. (2016) report that *Rubus fairholmianus* root extracts inhibit MCF-7 cells growth via caspase 3/7-induced apoptosis [45]. Reis et al. (2013) report that *Leccinum vulpinum* induces DNA damage, decreases cell proliferation and induces apoptosis in MCF-7 cells [46]. Miris et al. (2011) report that pomegranate (*Punica granatum* L.), at certain concentration, inhibits MCF-7 cell proliferation and induces increased expression of the pro-apoptotic gene Bax and decreased the expression of the anti-apoptotic gene Bcl-2. [47]. ASE extracts of grape leaves grown in Algeria were not cytotoxic for HUVEC cells. Atmaca et al. (2016) report that *Salvia triloba* L. extract has pro-apoptotic and anti-angiogenic effect in prostate cancer cell lines while being not cytotoxic for normal cells [48]. Aghbali et al. (2013) describe the pro-apoptotic potential of grape seeds extracts, confirmed by a significant inhibition of cell growth and viability in a dose- and time-dependent manner without inducing damage to HUVEC non-cancerous cells [49]. Indeed, the bioactive phytochemicals, Honokiol and Magnolol contained in *Magnolia officinalis* and their derivatives show an antiproliferative effect on HepG2 cell proliferation while being unable to elicit any effect on fibroblasts [50].

Finally, the literature strongly suggests that grape is a potential source of antioxidant, anticancer and cancer chemo-preventive phytochemicals. The other parts of the grapes, the skin and seeds, the whole grape by itself, grape-derived raisins and phytochemicals within the grapes have also been found to bear potential anticancer properties in various preclinical and clinical studies [51].

4. Materials and Methods

4.1. Chemicals and Cells

All solvents used were HPLC (High Performance Liquid Chromatography) grade purified (Merck, Darmstadt, Germany); water was purified using a milli-Qplus system from Millipore (Milford, MA, USA). Reagents employed were of analytical grade; Folin-Ciocalteu reagent and Sodium Carbonate (Na_2CO_3) were purchased from Carlo Erba (Milan, Italy); DPPH (2,2-diphenyl-1-picryhydrazyl) and gallic acid (3,4,5-trihydroxybenzoic acid) were purchased from Sigma-Aldrich, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) spin trap and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Milan, Italy).

HepG2 and MCF-7 cells were obtained from the Hospital of Cagliari, 09121, Cagliari, Italy. HUVECs cells were obtained from Gibco™ (Grand Island, NY, USA). Cisplatin was obtained from the Oncological Services Hospital of Sassari, Italy.

Dulbecco's phosphate buffered saline (DPBS) was purchased from Euroclone (Milano, Italy); Dulbecco's modified Eagle's Medium with phenol red (DMEM) and fetal bovine serum (FBS) from Life Technologies (Grand Island, NY, USA); Medium 200 and LSGS (5-003-10) from Gibco™. TRIzol reagent, SuperScript® VILO™ cDNA Synthesis Kit, Platinum Quantitative PCR Supermix UDG Kit, SybrGreen I, primer and fluorescein from Life Technologies (Grand Island, NY, USA). L-glutamine, Penicillin, Streptomycin, nonessential amino acids from Euroclone (Milano, Italy). (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction MTT Cell Proliferation Assay ATCC® 30-1010K kit was purchased from Invitrogen Co. All the primer sequences are represent in Table 3.

Table 3. Primers sequences used for real-time PCR reactions.

Primers	Forward	Reverse
hGAPDH	GAGTCAACGGATTTGGTCGT	GACAAGCTTCCCGTTCTCAG
BAX	TCTGACGGCAACTTCAACTG	TTGAGGAGTCTCACCCAACC
BCL-2	AGGATTGTGGCCTTCTTTGA	ACAGTTCCACAAAGGCATCC

4.2. Plant Material

Mature leaves from the *Vitis vinifera* L. apical portion were collected in Medea, Algeria in August. Leaves were rinsed with tap water and dried at room temperature (25 ± 3 °C). Finally, they were ground into a fine powder and kept in the dark at 5 °C in a sterile bag and under vacuum for further use.

4.3. Extraction Procedure

ASE was performed on a Dionex ASE 350 (Dionex Thermo FisherScientific Inc., Waltham, MA, USA). Powdered leaves (1 g) were weighed into a 22 mL Dionex (ASE 350) stainless-steel cell. The cells were equipped with a stainless-steel fit and a cellulose filter. The optimized operating conditions for ASE extraction are indicated in Table 4.

Table 4. Conditions of ASE extraction procedure.

Temperature (°C)	**40**
Pressure (PSI)	1500
Number of Cycle	2
Extraction time of one cycle (min)	5
Concentration of Ethanol (%)	60 Ethanol/40 water
Type of water used	Ultrapure

Two solvents were tested: ethanol 60% (v/v) and water. The extraction was performed in quadruplicate. After the extraction process, water extracts were immediately freeze-dried whereas the ethanolic ones were first evaporated under a nitrogen flow to remove ethanol, then freeze dried. The freeze-dried extracts were weighed and stored at −80 °C until analysis. Accelerated solvent extraction was performed with the lowest extraction temperature to avoid the maximum degradation of thermolabile compounds.

The extraction yield was calculated as follows:

$$\text{Yield\%} = \frac{(\text{the weight of freeze} - \text{dried recover})}{1 \text{ gram } (\text{initial weight of leaf powder used})} \times 100 \quad (1)$$

4.4. Total Phenolic (TP) Content

The total phenolic content was measured using the modified Folin-Ciocalteu method [52–54]. 1 mg of each lyophilized extract was mixed with 9 mL of cold ethanol (80%) (1:10 w/v), vortexed (Stuart, U.K. model SA8.) at 1600 rpm for 2 min and centrifuged (ALC-Centrifuge 4227R, Milan, Italy) at 16,000× g for 15 min at 4 °C. 200 µL of each extract were mixed with the Folin-Ciocalteu reagent (1 mL) and allowed to react for 8 min before adding 800 µL of sodium carbonate solution (0.075 mL^{-1}). The mixture was incubated in the dark for one hour at room temperature (20 ± 3 °C) followed by an additional hour at 0 °C. The absorbance was read at 760 nm with a spectrophotometer (8453 Agilent Technologies, Santa Clara, CA, USA).

Results were expressed as milligrams of gallic acid equivalent/g of dry weight on the basis of a gallic acid calibration curve (50 to 500 mg/L with R^2 = 0.996).

4.5. Antioxidant Activity

4.5.1. Spin Trapping Assay of the •OH Radical

The hydroxyl radical scavenging activity was determined with the spin trapping method coupled with electron paramagnetic resonance spectroscopy according to Fadda et al. [34]. The hydroxyl radicals were generated by the Fenton reaction and trapped with a nitrone spin trap 5,5-dimethyl-pyrroline N-oxide (DMPO) [55]. 20 mg of the freeze-dried extract mixed with 1 mL of

ultrapure water degassed under nitrogen flow was prepared as stock solution. Serial dilutions were prepared from the stock solution, and depending on the results, the correct concentration for each extract was established. 100 µL of the diluted samples were mixed with Fe(II) sulfate 0.1 mM (100 µL), 112 µL DMPO 26 mM (112 µL) and H_2O_2 1 mM (100 µL).

The DMPO-OH adduct was detected with a Bruker EMX EPR spectrometer operating at the X-band (9.4 GHz) using a Bruker Aqua-X capillary cell. The EPR instrument was set under the following conditions: modulation frequency, 100 kHz; modulation amplitude, 1 G; receiver gain, 1×10^5; microwave power, 20 mW. EPR spectra were recorded at room temperature immediately after the preparation of the reaction mixture. The concentration of the spin adduct DMPO-OH was estimated from the double integration of spectra. The hydroxyl radical scavenging activity was expressed as IC50 on the basis of the percentage of inhibition calculated as follows:

$$\%\ \text{inhibition} = \frac{(A_0 - A_s)}{A_0} \times 100 \quad (2)$$

where A_0 is the intensity of the spin adducts without extract and A_s is the absorbance of the adduct after the reaction with the extract. Different sample's concentrations were used to calculate the IC50, that is, the extract concentration that halves the concentration of hydroxyl radical adduct of the blank. Three replications were performed for each dilution.

4.5.2. DPPH

The radical scavenging activity of ethanolic and water extracts of grape leaves was determined spectrophotometrically with the DPPH test [56].

30 µL ASE ethanolic and water crude extract at different concentrations (0.05, 0.1, 0.2 mg mL^{-1}) were mixed with 3 mL of a DPPH methanol solution (0.3 mM). A blank solution was prepared using methanol instead of the extract.

Solutions were stored in the dark at room temperature for 30 minutes. The absorbance was measured at 518 nm and converted into the percentage of inhibition using the following equation:

$$\%\ inhibition = \frac{A_0 - A_s}{A_0} \times 100 \quad (3)$$

where A_0 is the absorbance of the sample without extract and A_S is the absorbance of the sample after the reaction with the extract. The DPPH radical scavenging activity was expressed as IC50. Three replications were performed for each dilution.

4.6. Cell Culture

HepG2 and MCF-7 cells were maintained in Dulbecco's modified Eagle's Medium with phenol red (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 200 of µM L-glutamine, 200 U/mL of penicillin, 10 µg/mL of streptomycin and 0.1 mM of non-essential amino acids. HUVEC cells were cultured in Medium 200 (Gibco™), containing LSGS (5-003-10; Gibco™), 200 U/mL of penicillin and 10 µg/mL of streptomycin. Cells were grown in 75 cm^2 tissue culture flasks in the culture incubator at 37 °C with 5% CO_2 and saturated humidity.

4.7. MTT Viability Assay

The anti-proliferative activity of ethanolic and aqueous ASE extracts of *Vitis vinifera* L. leaves on HepG2, MCF-7 and HUVEC cells was determined using a cell viability test.

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] tetrazolium reduction assay is a colorimetric assay based on the ability of functional mitochondria to reduce by succinate dehydrogenase enzyme an insoluble formazan crystal, which displays a purple color [57].

Then, the effects of the treatments on the overall growth of a particular cell population were assessed by determining the number of living cells remaining in the analyzed cell culture. After counting, HepG2, MCF-7 and HUVEC cells were seeded on a 96-well plate at concentration of 10,000/well in 200 µL and incubated at 37 °C in a 5% CO_2 incubator (Thermo Fisher Scientific, Waltham, MA, USA).

After 24 h, the medium was replaced with fresh medium containing compounds tested (ethanol and aqueous ASE crude extract) at concentration of 0.5 mg/mL, 1 mg/mL and 2 mg/mL. The negative control is performed in growing medium but positive control is prepared in medium supplemented with cosplatin 10 µM. Every test was repeated three times. After one day, we again substituted medium with or without compounds and repeated the same treatment (treatment 2). The MTT substrate was prepared in a sterile Dulbecco's phosphate buffered saline (DPBS), then added to cells in culture at a final concentration of 650 µg/mL and incubated for 3 h in the culture incubator at 37 °C with 5% CO_2 and saturated humidity. After incubation, the medium was removed by aspiration and 200 µL/well Dimethylsufoxide DMSO (Sigma Aldrich) was added to each well. Absorbance was read at 570 nm in a Gemini EMMicroplate Reader (Molecular devices). The percentage of cell proliferation was calculated relative to control wells designated as 100% viable cells using the following formula:

$$\frac{(\mathrm{At}-\mathrm{Ab})}{(\mathrm{Ac}-\mathrm{Ab})} \times 100 = \%\ cell\ proliferation \quad (4)$$

where At = absorbance value of test compound (ASE extract), Ab = absorbance value of blank (medium alone), Ac = absorbance value of control.

4.8. Gene Expression

HepG2 and MCF-7 cells were plated into 24-well cell culture plates (60,000 cells/500 µL for each well) in culture medium with ethanolic and aqueous ASE extracts of grape leaves to evaluate the expression levels of apoptotic-related genes. Extracts were prepared fresh just before each experiment and dissolved in DMEM.

After treatment, the total RNA was isolated using TRIzol reagent and quantified by measuring the absorbance at 260/280 nm (NanoDrop 2000, spectrophotometer Thermoscientific ND8008, Thermo Fisher Scientific, Waltham, MA, USA). Approximately 1 µg of total RNA was reverse-transcribed to cDNA by SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies, Grand Island, NY, USA).

Quantitative polymerase chain reaction was run in triplicate using a CFX Thermal Cycler (Bio-Rad, Hercules, CA, USA). 2 µL of cDNA were amplified in 25 µL reactions using Platinum Quantitative PCR Supermix UDG Kit. A Supermix 2X was mixed with Sybr Green I, 0.1 µM of primer and 10 nM fluorescein (Life Technologies, Grand Island, NY, USA). Relative target Ct (the threshold cycle) values of Bcl-2 and Bax were normalized to GAPDH, as housekeeping gene. The mRNA levels of cells treated with ethanolic and aqueous ASE extract were expressed using the $2^{-\Delta\Delta Ct}$ method [58], relative to the mRNA level of the untreated sample for each experiment.

4.9. Statistical Analysis

Results are expressed as mean ± standard deviation (SD) and were analyzed by ANOVA with Duncan's multiple range tests procedure (DMRT) and Student's t-test using 25.0 SPSS Windows software. Differences were considered significant for $p < 0.05$.

5. Conclusions

In the present study, we revealed for the first time that accelerator solvent extraction yielded a higher extraction rate of total phenols and antioxidant activity in an extremely short time. The extract obtained from grape leaves grown in the Medea region (Algeria) exhibited an antiproliferative effect on MCF-7 breast cancer cells and HepG2 hepatocarcinoma cells. Moreover, considering previous

reports by other authors and the present results that provide evidence for the modulation of Bax/Bcl2 mRNA levels by leaf extracts, which affects the balance between apoptosis and cell survival, it may be concluded that these extracts could be used as an easily accessible source of natural antioxidants, and as a matrix to prepare drugs counteracting distinctive cancer cells' proliferation.

Author Contributions: S.F. is the leading author, who developed most of the idea and wrote the article. S.S., S.C., A.F. and D.S. provided technical guidance during simulations and experiments; M.M. provided the necessary technical tools for the realization of this work and followed the writing of the paper and its revision. S.Z. is the supervisor of this work. A.D. and G.D. supervised the work and are the coordinators of the guesting Institute.

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Sample Availability: Samples of the compounds from grape leaves are available from the authors.

Abstract

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Oxidative stress is defined as an imbalance between productions of free radicals and reactive metabolites. This imbalance leads to damage of important biomolecules and cells, with potential impact on the whole organism. There is currently arenewed interest in phytochemicals as sources of natural antioxidants.

Hyperhomocysteinemia is usually defined as an elevation of plasma tHcy. It is an important risk factor for premature cardiovascular disease. As a result, homocysteine metabolism abnormalities are now receiving increasing attention because of their potential role in the pathogenesis of atherosclerosis and other diseases such as venous thrombosis.

In this study, we tested *in vivo* the antioxidant and anti-inflammatory effect of the Grape Leaves (*Vitis vinifera*) Algerian Variety on the cardiovascular inflammation induced by Hyperhomocysteinemia. The phenolic compounds (Anthocyanins and non anthocyanins) is identified by using HPLC-DAD/ESI-MS, total Homocysteine (t-Hcy) was estimated by competitive solid phase chemiluminescence immunoassay, the plasma hs-CRP is measured by immunoturbidimetric method and the concentration of the GSH measured by spectrophotometric method. In addition, the aorta histology examined in order to confirm the angiotoxic action of homocysteine and the reparative effect of grape leaves (*Vitis vinifera*) on the aorta.

Results showed high levels of phenols, anthocyanins, flavonols and trans-caftaric acid in grape leaves (*Vitis vinifera*). The plasma hs-CRP and homocysteine levels were elevated significantly ($p < 0.05$) however the glutathione reduced significantly ($p < 0.05$) after the administration of L-methionine in high doses to mice. This was associated with the desquamation of endothelium and muscular lysis with transformation of spindle nuclei to oval nuclei; this is due to the angiotoxic action of homocysteine on the aorta. These changes were not observed in mice treated with L-methionine plus the antioxidant and anti-inflammatory extract of grape leaves (*Vitis vinifera*). So, the study proved the antioxidant and anti-inflammatory effects of the grape leaves (*Vitis vinifera*) on hyperhomocysteinemia induced inflammatory endothelial damage in cardiovascular diseases.

Moreover, in the present thesis, we conducted a study *in vitro* to analyze the total phenols, anti-oxidative, anti-proliferative properties on MCF-7 and HepG2 Human Cancer Cell Lines and on modulate of Apoptosis-Related Gene Expression of water and ethanol crude extracts obtained by Accelerator Solvent Extractor from grape leaves (*Vitis vinifera*) grown in Algeria.
The results revealed for the first time that accelerator solvent extraction yielded a higher extraction rate of total phenols and antioxidant activity in an extremely short time. The extract obtained from grape leaves (*Vitis vinifera*) grown in the Medea region (Algeria) exhibited an antiproliferative effect on MCF-7 breast cancer cells and HepG2 hepatocarcinoma cells. Moreover, considering previous reports by other authors and the present results that provides evidence for the modulation of Bax/Bcl2 mRNA levels by leaf extracts, which affects the balance between apoptosis and cell survival, it may be concluded that these extracts could be used as an easily accessible source of natural antioxidants, and as a matrix to prepare drugs counteracting distinctive cancer cells' proliferation.

Keywords: Grape leaves *Vitis vinifera*, HPLC, phenols, Homocysteine, hs-CRP, GSH, ASE, DPPH, ERP, MCF-7, HepG2, HUVEC, Bax/Bcl-2**.**

Résumé

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Le stress oxydatif est défini comme le déséquilibre entre la production des radicaux libres et des métabolites réactifs. Ce déséquilibre peut entraîner des dommages sur l'ensemble de l'organisme. Afin de se protéger contre ces effets, on constate actuellement un regain d'intérêt pour les substances phytochimiques comme sources d'antioxydants naturels.
L'hyperhomocystéinémie est habituellement définie comme une élévation de la tHcy plasmatique. Il s'agit d'un important facteur de risque des maladies cardiovasculaires prématurées. Par conséquent, les anomalies du métabolisme de l'homocystéine font l'objet d'une attention croissante en raison de leur rôle potentiel dans la pathogenèse de l'athérosclérose et d'autres maladies comme la thrombose veineuse.
Dans cette étude, nous avons testé *in vivo* l'effet antioxydant et anti-inflammatoire des feuilles de vigne (*Vitis vinifera*) la variété algérienne sur l'inflammation cardiovasculaire induite par l'hyperhomocystéinémie. Les composés phénoliques (anthocyanines et non anthocyanines) sont identifiés par HPLC-DAD/ESI-MS, le taux de l'homocystéine, le plasma hs-CRP et la concentration du GSH sont mesurés. L'histologie des différents étages de l'aorte des souris traitées a été examinée afin de confirmer l'action angiotoxique de l'homocystéine et l'effet réparateur des feuilles de vigne sur l'aorte.
Les résultats ont montré des niveaux élevés de phénols, d'anthocyanines, de flavonols et d'acide trans-caftarique dans les feuilles de *Vitis vinifera* la variété algérienne. Les taux plasmatiques de hs-CRP et d'homocystéine étaient significativement élevés ($p < 0,05$), mais le glutathion a diminué significativement ($p < 0,05$) après l'administration de L-méthionine à fortes doses aux souris. Ceci était associé à la desquamation de l'endothélium et à la lyse musculaire avec transformation des noyaux fuseau en noyaux ovale. Les altérations observées sont dûes à l'action angiotoxique de l'homocystéine sur l'aorte. Ces changements n'ont pas été observés chez les souris traitées par la L-méthionine et les feuilles de *Vitis vinifera.* L'étude a prouvé les effets antioxydants et anti-inflammatoires des feuilles de *Vitis vinifera* sur les dommages endothéliaux et inflammatoires induits par l'hyperhomocystéinémie dans les maladies cardiovasculaires.
De plus, nous avons mené une étude *in vitro* pour doser les polyphénols totaux, l'activité antioxydantes et antiprolifératives sur des lignées cellulaires cancéreuses humaines MCF-7 et HepG2 et sur la modulation d'expression génique des extraits bruts aqueux et éthanoliques obtenus par Extraction Accélérée par Solvant (ASE) à partir des feuilles de vigne (*Vitis vinifera*) cultivées en Algérie.
Les résultats ont révélé pour la première fois que l'extraction par l'ASE permettait d'obtenir un taux d'extraction plus élevé des phénols totaux dans un temps extrêmement court et une activité antioxydante élevée. L'extrait obtenu à partir de feuilles de vigne (*Vitis vinifera*) cultivées en Algérie a montré un effet antiprolifératif sur les cellules cancéreuses du sein MCF-7 et les cellules hépatocarcinomes HepG2. En compte tenu des rapports précédents d'autres auteurs et des résultats actuels qui fournissent des preuves de la modulation des niveaux d'ARNm Bax/Bcl2 par des extraits de feuilles de *Vitis Vinifera,* ce qui affecte l'équilibre entre l'apoptose et la survie cellulaire. Finalement, on peut conclure que les extraits des feuilles de *Vitis vinifera* pourraient être utilisés comme source facilement accessible d'antioxydants naturels et comme source pour préparer des médicaments qui combattent la prolifération des différentes cellules cancéreuses.

Mots-clés : Feuilles de vigne *Vitis vinifera*, HPLC, phenols, Homocysteine, hs-CRP, GSH, ASE, DPPH, ERP, MCF-7, HepG2, HUVEC, Bax/Bcl-2**.**

ملخص

ملخص

إن الإجهاد التأكسدي هو عبارة عن عدم التوازن ما بين إنتاج الجذور الحرة ومضادات الأكسدة، يؤدي هذا الخلل إلى تلف الخلايا والجزيئات الحيوية الهامة مع إحتمال التأثير على العضوية كلها. يوجد حاليا إهتمام بالكمياء النباتية كمصدر لمضادات الاكسدة التي يمكن أن تقي من هذه الجذور الحرة المتسببة في عدة أمراض.

عادة ما يعرف فرط الهوموسستيين البلازمي بزيادة الهوموسستيين الكلي في بلازما الدم، الذي يعتبر عامل خطير لأمراض القلب والأوعية الدموية. نتيجة لذلك، فإن الخلل في أيض الهوموسستيين تلقى إهتماما كبيرا بسبب دوره في حدوث تصلب الشرايين وغيرها من الأمراض مثل تجلط الدم الوريدي.

خلال هذه الدراسة تم تقييم داخل العضوية تأثير مضادات الأكسدة والإلتهابات لأوراق العنب على القلب والأوعية الدموية الناجمة عن زيادة الهوموسستيين البلازمي في الجسم وذلك بتحديد المركبات الفنيولية ، الهوموسستيين الكلي، بروتين(hs-CRP) والجلوتاثيون المختزل (GSH).

بالإضافة إلى ذلك تم دراسة نسيجية للآورطي وذلك لمعرفة مدى تأثير سمية زيادة الهوموسستيين البلازمي ومعالجته بواسطة مستخلص أوراق العنب (*Vitis vinifera*). أظهرت النتائج بأن مستخلص أوراق العنب تحتوي على كميات عالية من الفينولات، الأنثوسيانين، الفلافونول وحمض الكافتريك كما لوحظت زيادة في تراكيز بروتين hs-CRP ، الهوموسستيين بقيمة معتبرة مع ظهور إنخفاض الجلوتاثيو GSH المختزل بقيمة معتبرة $P<0,05$عند الفئران المتغذية على جروعات عالية من المثيونين كما لوحظ تقشر في البطانة الداخلية للآورطي، تحلل في الخلايا العضلية المكونة للطبقة المتوسطة للآورطى مع تغيير في شكل نواة الخلايا العضلية من الشكل المغزلي للشكل الدائري .

بينما تحصلنا على تراكيز منخفضة للبروتينhs-CRP ، الهوموسستيين مع زيادة في الجلوتاثيون GSH وظهور قطاعات نسيجية عادية عند الفئران المعاملة بواسطة المثيونين بجرعة 1غ/ كلغ ومستخلص المضاد للأكسدة والإلتهاب لأوراق العنب 500ملغ/ كلغ لمدة 15يوم.

إن مستخلص أوراق العنب المستخدمة في هذه الدراسة لها تأثير مضاد للأكسدة والإلتهابات المتسببة بواسطة زيادة الهوموسستيين البلازمي عند الفئران.

بالإضافة إلى ذلك، أجرينا دراسة خارج العضوية على تقييم الفينولات الكلية المضادة للأكسدة على تكاثر الخلايا السرطانية للإنسان و المتمثلة في خلايا سرطان الثدي (MCF-7) وخلايا سرطان الكبد (HepG2) وعلى الجنيات Bax و Bcl-2 لهذه الخلايا السرطانية مع مقارنتها بالخلايا الطبيعية للخلية البطانية من الوريد السري (HUVEC) لمستخلص المائي والإثانولي لأوراق العنب المتحصل عليه بواسطة مستخرج المذيبات المعجل(ASE).

أظهرت النتائج بأن مستخلص أوراق العنب (المائي والإيثانولي) أدى الى زيادة في جينات النسخ Bax وانخفاض في Bcl-2 في كل من الخلايا السرطانية للثدي والكبد .إن النتائج المتحصلة عليها في هذه الدراسة بينت بأن مستخلص أوراق العنب(*Vitis vinifera*) يعتبر مصدرا طبيعيا يمكن استخدامه في الوقاية من أمراض القلب والأوعية الدموية والسرطانية.

الكلمات المفتاحية : أوراق العنب (*Vitis vinifera*)، HPLC، فينولات، الهوموسستيين، hs-CRP، الجلوتاثيون المختزل GSH، مستخرج المذيبات المعجل(ASE) ، مستقر الجذور الحرة DPPH، الكترون رنين مغنطيسي (ERP)، خلايا سرطان الثدي (MCF-7) ، خلايا سرطان الكبد (HepG2) ، الخلية البطانية من الوريد السري(HUVEC) ، الجنيات Bax/Bcl-2.

Family Name: FERHI First Name: Selma	Academic year: 2018-2019

Biological activities of *Vitis vinifera* leaves in cardiovascular diseases induced by hyperhomocysteinemia and on tumoral process

Thesis submitted for the degree of Doctorat Science in biological sciences and biotechnology

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