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).

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.

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.

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 $[M\text{-}H\text{-}162]^-$ (quercetin-3-galactoside/glucoside) and $[M\text{-}H\text{-}176]^-$ (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.

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.
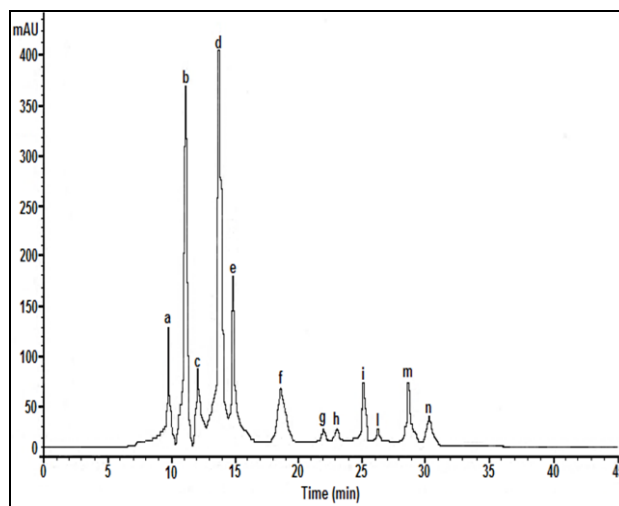

Figure 1. *HPLC-DAD Chromatogram at 530 nm of anthocyanin compounds from a Vitis vinifera L. leaves methanol extract. (a) delphinidin 3-glucoside; (b) cyanidin 3-glucoside; (c) petunidin 3-glucoside; (d) peonidin 3-glucoside; (e) malvidin 3-glucoside; (f) cyanidin 3-(6-p-acetyl) glucoside; (g) peonidin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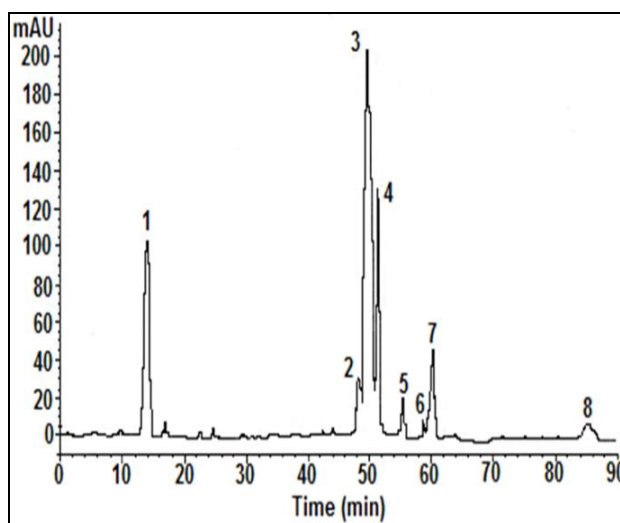

Figure 2. *HPLC-DAD Chromatogram at 340 nm of non-anthocyanin compounds from a Vitis vinifera L. leaves methanol extract. (1) trans-caftaric acid; (2) Quercetin-3-O-galactoside; (3) quercetin 3-0-glucuronide; (4) quercetin 3-0-glucoside; (5) kaempferol 3-0-galactoside; (6) kaempferol 3-0-glucuronide;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

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).

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

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	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

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.

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 ($P > 0.05$).
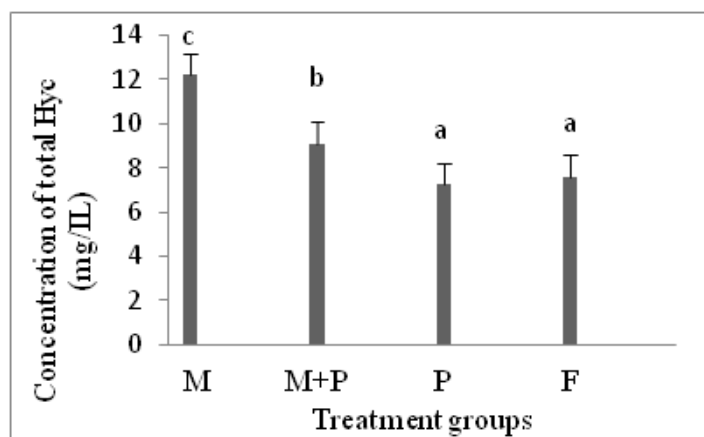
Figure 3. *Total Hcy level in the groups treated during 15 days. Values are the means ± SEM (n)*

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).
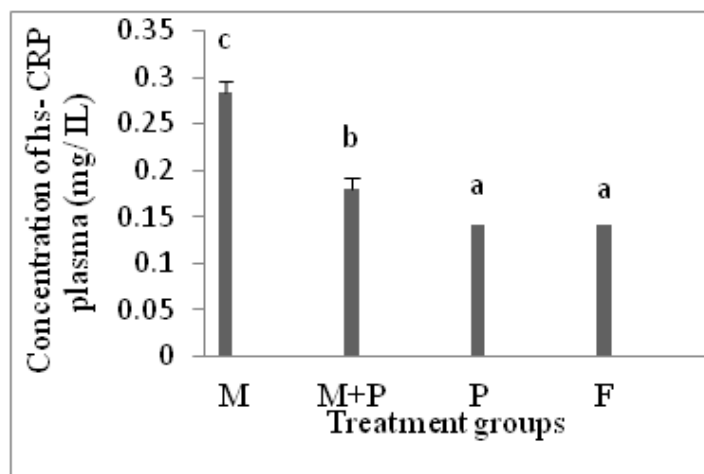

Figure 4. *Concentration of hs-CRP plasma in the groups treated during 15 days. Values are the means ± SEM (n)*

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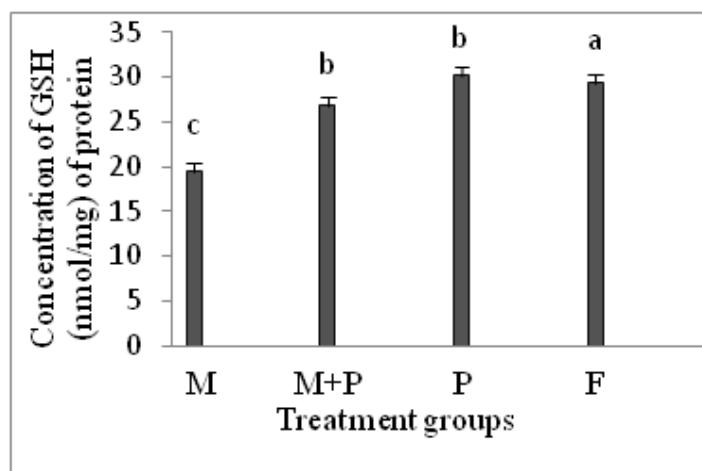
Figure 5. *Concentration of GSH in the groups treated during 15 days. Values are the means ± SEM (n)*

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*).
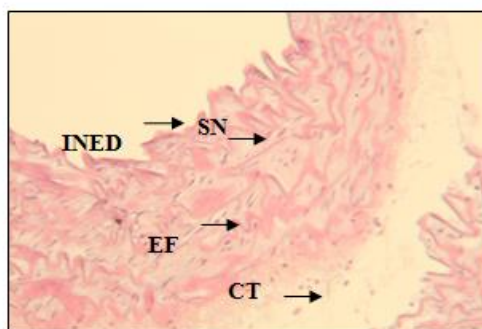

Figure 6. *Longitudinal section of arch aorta 15 days of flour application. Heamatoxylin Eosin staining (x100). EF: elastic fiber, SN: spindle nuclei, CT: connective tissue, INED: intact endothelium*
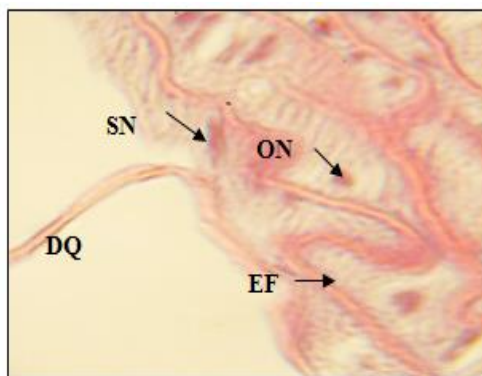
*Figure 7. Longitudinal section of arch aorta 15 days of L-methionine application.
Heamatoxylin Eosin staining (x400). EF: elastic fiber, DQ: desquamation, ON: oval nuclei, SN:
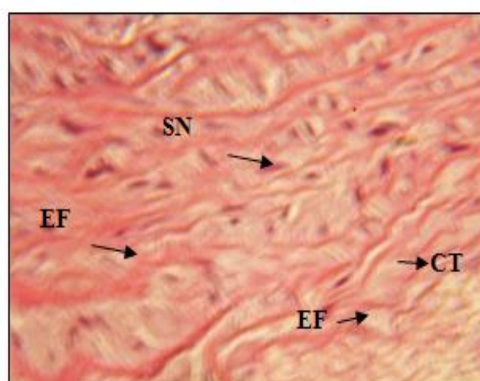spindle nuclei*

*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

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 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 et al., 2001). Yalçinkaya et al. (2009) demonstrated that a high methionine diet induced oxidative stress in serum, heart, and aorta in rabbits.

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.

REFERENCES

[1]   Benmebarek, A., Zerizer, S., Laggoune, S., Kabouche, Z. (2013): Effect of *Stachysmialhesi* de Noé on the inflammation induced by hyperhomocysteinemia in cardiovascular diseases. – Der Pharmacia Lettre 5(2): 212-223.

[2]   Bernardo Aguilar, M. D., Julio, C., Rojas, M. D., Collados, M. T. (2004): Metabolism of homocysteine and its relationship with cardiovascular disease. – Journal of Thrombosis and Thrombolysis 18(2): 75-87.

[3]   Boldyrev, A. A. (2009): Molecular mechanisms of homocysteine toxicity. – Biochemistry (Moscow) 74(6): 589-598.

[4]   Chiang, E. P., Smith, D. E., Selhub, J. et al. (2005): Inflammation causes tissue-specific depletion of vitamin B6. – Arthritis Res Ther 7: R1254-62.

[5]   Dani, C., Oliboni, L. S., Agostini, F., Funchal, C., Serafini, L., Henriques, J. A., Salvador, M. (2010): Phenolic content of grapevine leaves (Vitislabrusca var. Bordo) and its neuroprotective effect against peroxide damage. – Toxicology in Vitro 24: 148-153.

[6]   Demuth, K., Drunat, S., Paul, J.-L., Moatti, N. (2000): Hyperhomocystéinémie et athérosclérose medicine. – Sciences 16: 1081-90.

[7]   Dobes, J., Zitka, O., Sochor, J., Ruttkay-Nedecky, B., Babula, P., Beklova, M., Kynicky, J., Hubalek, J., Klejdus, B., Kizek, R., Adam, V. (2013): Electrochemical tools for determination of phenolic compounds in plants. – Int. J. Electrochem. Sci. 8: 4520-4542.

[8]   Falvoa, N., Ghiringhellib, F., Berthiera, S., Bonnottea, B., Lorceriea, B. B. (2007): Case-control study of hyperhomocysteinemia in cancer-bearing patients in a internal medicine department. – La Revue de Médecine Interne 28: 520-525.

[9]   Flamini, R., De Rosso, M., Bavaresco, L. (2015): Study of grape polyphenols by liquid chromatography-high-resolution mass spectrometry (UHPLC/QTOF) and suspect screening analysis. –J. Analytical Methods in Chemistry 1-10.

[10]   Flemming, S., Mette, G., Mudd, S. H. (2010): A revisit to the natural history of homocystinuria due to cystathionine β-synthase deficiency. – Mol Genet Metab 99(1): 1-3.

[11]   Folsom, A. R., Aleksic, N., Catellier, D., Juneja, H. S., Wu, K. K. (2002): C-reactive protein and incident coronary heart disease in the Atherosclerosis Risk In Communities (ARIC) study. –Am Heart J 144: 233-238.

[12]   Fraga, C. G., Galleano, M., Verstraeten, S. V., Oteiza, P. I. (2010): Basic biochemical mechanisms behind the health benefits of polyphenols. – Mol Aspects Med. 31: 435-445.

[13]   Haleng, J., Pincemail, J., Defraigne, J. O., Charlier, C., Chapelle Fonc, J. P. (2007): Le stress oxidant. – Rev Med Liege 62(10): 628-638.

[14]   Hennebelle, T., Sahpaz, S., Bailleu, F. (2004): Polyphénols végétaux, sources, utilisations et potentiel dans la lutte contre le stress oxydatif. – Phytothérapie 1: 3-6.

[15]   Houssem Eddine, K., H., Zerizer, S., Kabouche, Z. (2014): Immunostimulatory activity of Phoenix Dactylifera. –International Journal of Pharmacy and Pharmaceutical Sciences 6(3): 73-76.

[16]   Jacobsen, D. W. (2000): Hyperhomocysteinemia and oxidative stress time for a reality check? – Arterioscler Thromb Vasc Biol 20: 1182-1184.

[17]   Kammerer, D., Claus, A., Carle, R., Schieber, A. (2004): Polyphenol screening of pomace from red and white grape varieties (Vitis vinifera L.) by HPLC-DAD-MS/MS. – J. Agric. Food Chem. 52: 4360-4367.

[18]   Monagas, M., Hernaändez-Ledesma, B., GoäMez-Cordoveä, C. S., Bartolomeä, B. A. (2006): Commercial dietary ingredients from Vitis vinifera L. leaves and grape skins: antioxidant and chemical characterization. – J. Agric. Food Chem. 54: 319-327.

[19]   Morand, C., Milenkovic, D. (2014): Polyphénols et santé vasculaire: mise en évidence du rôle direct des polyphénols dans les effets bénéfiques des agrumes dans la protection vasculaire. – Innovations Agronomiques 42: 47-62.

[20] Morris, M. S., Jacques, P. F., Van Den Berg, I. H. R., Selhub, J. (2001): Hyperhomocysteinemia associated with poor recall in the third National Health and Nutrition xamination Survey. – Am J Clin Nutr 73: 927-33.

[21] Rahman, I., Kode, A., Biswas, S. K. (2006): Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. – Nature Protocols 1(6): 3159-3165.

[22] Refsum, H., Ueland, P. M., Nygard, M. D., Vollset, S. E. (1998): Homocysteine and cardiovascular disease. – Annu. Rev. Medicine 49: 31-62.

[23] Rigaud, D. (1999): Hyperhomocystéinémie et maladies cardiovasculaires dues à l'athérosclérose. – La Lettre du Cardiologue - n° 309 - mars 1999. Service hépato-gastroentérologie et nutrition, CHU Bichat - Claude-Bernard, Paris.

[24] Stalder, M., Lovey, P.-Y., Dayer, E. (2007): Homocystéine et maladie thromboembolique. – Organe de publication pour l'institut Central des Hôpitaux Valaisans, Sion 9(3).

[25] Van Den Berg, M., Boers, G. H. J., Franken, D. G., Bom, H. J., Van Kamp, G. J., Jakobs, C., Rauwerda, J. A., Klift, C., Stehouwert, C. D. A. (1995): Hyperhomocysteinaemia and endotheliaI dysfunction in young patients with peripheral arterial occlusive disease. – European Journal of Clinical Investigation 25: 176-181.

[26] Villiers de, A., Vanhoenacker, G., Majek, P., Sandra, P. (2004): Determination of anthocyanins in wine by direct injection liquid chromatography-diode array detection-mass spectrometry and classification of wines using discriminant analysis. – J. Chroma. 1054: 195-204.

[27] Wang, G., O, K. (2001): Homocysteine stimulates the expression of monocyte chemo attractant protein-1 receptor (CCR2) in human monocytes: possible involvement of oxygen free radicals. – Biochemistry Journal 357: 233-240.

[28] Williams, F. F. et al. (2002): Coronary endothelial function in hyperhomocysteinemia: improvement after treatment with folic acid and cobalamin in patients with coronary artery disease. – J Am Coll Cardiol 40: 766-72.

[29] Wu, J. T. (2008): Circulating homocysteine is an inflammation marker and arisk factor of life-threatening inflammatory diseases. – Journal of Biomedical Science 19(4): 107-112.

[30] Yalçinkaya-Demirsöz, S., Depboylu, B., Dogru-Abbasoglu, S., Unlüçerçi, Y., Uysal, M. (2009): Effects of high methionine diet on oxidative stress in serum, apo-B containing lipoproteins, heart, and aorta in rabbits. – Annals of Clinical and Laboratory Science 39(4): 386-91.

[31] Zeng, X. K., Remick, D. G., Wang, X. (2004): Homocysteineinduces production of monocyte chemoattractant protein-1 and interleukin-8 in cultured human whole blood. – Acta Pharmacologica Sinica 25(11): 1419-1425.

[32] Zerizer, S. (2006): Hyperhomocysteinemia, B vitamins and atherogenesis. Clinical and experimentalstudies. – Thesis submitted for the degree of DOCTORAT D'ETAT in Natural Sciences Option: Animale physiology. Université de Constantine, pp. 33-34.

[33] Zerizer, S., Naimi, D. (2004): Homocysteine: An independent risk factor in atherogenic Process. – Egyptian Pharmaceutical Journal 3: 110-114.

[34] Zitoun, J. (1998): Plasma homocysteine levels related to interactions between folate status and ethyltetrahydrofolatereductase. A study in 52 healthy subjects. Metabolism. – Clinical and Experimental 47: 1413-8.