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Title: Chemo-protective activity and characterization of phenolic extracts from Corema album.

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Keywords: antioxidant defences; biomarkers of oxidative damage; chlorogenic acid; hydroxycinnamic acids; natural bioactive compounds; oxidative stress

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Abstract: There is currently substantial interest in the cyto-protective effects of natural compounds against oxidative stress and in studying of the defence mechanisms involved. Corema album fruit is an edible berry consumed along the Atlantic littoral of the Iberian Peninsula. The aim of this study was to characterize the phenolic composition and evaluate the chemo-protective effects against oxidative stress of three phenolic extracts from this fruit on liver cells.
Characterization of phenolic compounds, achieved by liquid chromatography and diode-array, mass spectrometry and electrospray ionization-time of flight-mass spectrometry detection, showed a main fraction of hydroxycinnamic acids. Liver HepG2 cells were treated with 1-40 μg/mL of the extracts and exposed to oxidative stress chemically induced. Cell viability, reactive oxygen species (ROS), reduced glutathione (GSH), antioxidant enzymes and biomarkers of oxidative damage were evaluated.
Treatment of HepG2 cells with the extracts partially prevented ROS increase, GSH depletion, antioxidant enzymes over-activity and oxidative damage to proteins and lipids induced by stress. The results support the traditional use of Corema album as a medicinal plant and suggest that inclusion of its berries in the diet would contribute to the protection afforded by fruits, vegetables and plant-derived beverages against oxidative stress related diseases.

July 5th, 2012

To: Editor of Food Research International

Dear Editor,

Please find enclosed the manuscript entitled: **Chemo-protective activity and characterization of phenolic extracts from *Corema album***, by Antonio León-González, Raquel Mateos, Sonia Ramos, M. Ángeles Martín, Beatriz Sarriá, Carmen Martín-Cordero, Miguel López-Lázaro, Laura Bravo and Luis Goya for submission to Food Research International. The results reported in this manuscript have not been submitted to any other journal.

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Manuscript significance

The results of the present study show that *Corema album* berry phenolic extracts are mainly rich in hydroxycinnamic acids and treatment with the extracts protects cultured liver cells against oxidative stress chemically induced.

Thanking you in advance. We look forward to hearing from you.

Yours sincerely,

Luis Goya

Department of Nutrition and Metabolism

ICTAN (CSIC)

Highlights

1) *Corema album* berry phenolic extracts are mainly rich in hydroxycinnamic acids
2) Treatment with the extracts directly improves redox condition of liver cells
3) Treatment with the extracts protects liver cells against oxidative stress

Chemo-protective activity and characterization of phenolic extracts from *Corema album*.

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Running title: *Corema album* hydroxycinnamic acids protect HepG2 cells from oxidative stress.

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Abstract

There is currently substantial interest in the cyto-protective effects of natural compounds against oxidative stress and in studying of the defence mechanisms involved. *Corema album* fruit is an edible berry consumed along the Atlantic littoral of the Iberian Peninsula. The aim of this study was to characterize the phenolic composition and evaluate the chemo-protective effects against oxidative stress of three phenolic extracts from this fruit on liver cells.

Characterization of phenolic compounds, achieved by liquid chromatography and diode-array, mass spectrometry and electrospray ionization-time of flight-mass spectrometry detection, showed a main fraction of hydroxycinnamic acids. Liver HepG2 cells were treated with 1-40 µg/mL of the extracts and exposed to oxidative stress chemically induced. Cell viability, reactive oxygen species (ROS), reduced glutathione (GSH), antioxidant enzymes and biomarkers of oxidative damage were evaluated.

Treatment of HepG2 cells with the extracts partially prevented ROS increase, GSH depletion, antioxidant enzymes over-activity and oxidative damage to proteins and lipids induced by stress. The results support the traditional use of *Corema album* as a medicinal plant and suggest that inclusion of its berries in the diet would contribute to the protection afforded by fruits, vegetables and plant-derived beverages against oxidative stress related diseases.

Keywords: antioxidant defences, biomarkers of oxidative damage, chlorogenic acid, hydroxycinnamic acids, natural bioactive compounds, oxidative stress.

1. Introduction

Oxidative stress is the main cause of liver diseases and plant extracts with antioxidant properties have received extensive attention as possible therapeutic and preventive agents which counteract the production of free radicals and reactive oxygen species (ROS) and thus combat oxidative stress.

Plant polyphenols have gained increasing interest because of their numerous biological effects such as free-radical scavenging, metal chelation, modulation of enzymatic activity, inhibition of cellular proliferation and altering signal transduction pathways (Scalbert et al., 2005). Epidemiological studies have also highlighted the association between the consumption of polyphenol-rich foods and the prevention of degenerative human diseases such as cardiovascular diseases, cancer and other degenerative disorders (Manach et al., 2004).

Corema album D. Don (Ericaceae) fruit is a wild edible berry traditionally consumed along the Atlantic littoral of the Iberian Peninsula. Berries *of Corema album* have been used in popular medicine as antipyretic and are offered in the south of Spain as appetizers. Unpublished results have indicated that the main phenolic fraction in *Corema album* is composed by hydroxycinnamic acids. The major hydroxycinnamic acids, *p*-coumaric, caffeic, ferulic and sinapinic acids, are ubiquitously found in fruits, vegetables, cereals and also in high concentrations in tea, mate (Bravo et al., 2007) and particularly in coffee, averaging 160 mg per cup (Williamson et al., 2011). The interest of these compounds is related to their antioxidant activity, which may have some health beneficial effects *in vivo*. The extent of their protective effect *in vivo* depends on their bioavailability for intestinal absorption, metabolism, and subsequent interaction with target tissues. In this line, different studies have shown that hydroxycinnamic acids are extensively absorbed in cultured cells (Mateos et al., 2006), rats (Azuma et al., 2000; Lafay et al., 2006) and humans (Nardini et al., 2002; Olthof et al., 2001).

The liver is particularly susceptible to toxic and oxidative insults since the portal vein brings blood to this organ after intestinal absorption. The absorbed drugs and xenobiotics can cause ROS- and free radical-mediated damage that may result in inflammatory and fibrotic processes (Lima et al., 2006). Therefore, studies dealing with the effects of antioxidants at a cellular level in cultured hepatic cells are essential. Human HepG2 is a cell culture model of human hepatocytes widely used for pharmacological studies since they retain their morphology and most of their function in culture (Alía et al., 2006). Different studies have demonstrated that hydroxycinnamic acids (Mateos et al., 2006), flavonoids (Kanazawa et al., 2006) and olive oil phenols, hydroxytyrosol and hydroxytyrosyl acetate (Mateos et al., 2005) are absorbed and metabolized by cultured HepG2 cells. In this study, the main phenolic compounds in three different extracts from *Corema album* were characterized and quantified, and their hepato-protective activity against an oxidative challenge was tested in HepG2 cells.

2. Materials and Methods

2.1. Reagents

Formic acid and methanol grade HPLC were obtained from Panreac (Barcelona, Spain). Chlorogenic acid, rutin, hesperetin, resveratrol and *p*-hydroxybenzoic acid, *tert*-butylhydroperoxide (*t*-BOOH), *o*-phthalaldehyde (OPT), glutathione reductase (GR), reduced (GSH) and oxidized glutathione, nicotine adenine dinucleotide (reduced) (NADH), nicotine adenine dinucleotide phosphate reduced salt

(NADPH), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), 2,4-dinitrophenylhydrazone (DNPH), gentamycin, penicillin G, streptomycin, β-mercaptoethanol and EDTA were purchased from Sigma-Aldrich (Madrid, Spain). Cyanidin 3-*O*-glucoside chloride, delphinidin 3-*O*-glucoside chloride and cyanidin 3-*O*-arabinoside chloride were acquired from Extrasynthese (Lyon, France). The Bradford reagent was from BioRad Laboratories (Madrid, Spain). Other reagents were of analytical or chromatographic quality.

2.2. Extraction of Phenolic Compounds from Corema album

Wild *Corema album* berries were harvested in Huelva (Spain) 37º 04´10.15" N - 6º 41´15.45" W, in September 2009, and identified by Dr. Mari Cruz Diaz Barradas, from Department of Plant Biology and Ecology, University of Seville. *Corema album* fruits not showing any physical damage were selected, washed under running tap water and blot dried. Ripe fruits were lyophilized and freeze-dried samples were ground and stored at −20 °C until further analysis. Three different phenolic compounds extraction methods were applied: 'Acetone extract' (A) was obtained by homogenizing 100 g of lyophilized *Corema album* with 100 mL of acetone/formic acid/water (70:0.5:29.5, v/v/v) using ultrasonic equipment for 45 min at room temperature. The final extract was lyophilized and resulting a 4.79% yield with respect to fresh fruit. 'Ethyl acetate extract' (EA) was obtained by homogenizing 100 g of lyophilized fruit with 100 mL of ethyl acetate using ultrasonic equipment for 45 min at room temperature. The final extract was evaporated under vacuum producing a 0.29% yield with respect to fresh fruit. 'Water extract' (W) was obtained by homogenizing 300 g of frozen ripe fruit with 300 mL of water using ultrasonic equipment for 45 min at room temperature. The final extract was lyophilized and resulting a 4.5% yield with respect to fresh fruit. Finally, 10 mg/mL stock solutions of A and W in water and EA in ethanol were prepared to characterize their phenolic composition by HPLC and for cell treatment.

2.3. HPLC Analysis

Phenolic composition of extracts was analyzed using an Agilent 1100 liquid chromatographic system equipped with an autosampler, quaternary pump and diode-array (DAD) detector. A 250 mm x 4.6 mm i.d., 5-μm particle size Nucleosil 120 RP-18 column (Teknokroma) preceded by a ODS precolumn was used. Elution was performed at a flow rate of 1.0 mL/min, using as mobile phase mixture of 1% (v/v) formic acid in deionized water (solvent A) and methanol (solvent B). The solvent gradient changed from 90% A to 85% A in 5 min, to 70% A in 15 min, to 50% A in 15 min, to 30% A in 7 min, to 10% A in 3 min maintained for 5 min and to 90% A in 10min. Chromatograms were acquired at 280 nm to register hydroxybenzoic acids, flavanones and stilbenes. Wavelengths 320, 360 and 520 nm were selected to monitor hydroxycinnamic acids, flavonols and anthocyanins, respectively. *p*-hydroxybenzoic acid, hesperetin, resveratrol, chlorogenic acid, rutin and cyanidin 3-*O*-glucoside were used to quantify hydroxybenzoic acids, flavanones, stilbenes, hydroxycinnamic acids, flavonols and anthocyanins, respectively

2.4. LC-MS Analysis

LC-MS measurements were performed on an Agilent 1100 series liquid chromatograph/mass selective detector equipped with a DAD detector and a quadrupole mass spectrometer (Agilent Technologies). Chromatographic conditions (eluents, column, flow rate, gradient, etc.) were as described above. Eluent flow was split 8:1 between the DAD detector and the MS ion source. The MS was fitted to

an atmospheric pressure electrospray ionization (ESI) source, operated in negative ion mode. The electrospray capillary voltage was set to 3000 V, with a nebulizing gas (nitrogen) flow rate of 12 L/h and a drying gas temperature of 300 °C. Mass spectrometry data were acquired in scan mode (mass range m/z 100-900) at a scan rate of 1.5 s.

2.5. HPLC-ESI-QTOF Analysis

The chromatography was performed on an Agilent 1200 series LC system coupled to an Agilent 6530A Accurate-Mass Quadrupole Time-of-Flight (Q-ToF) with ESI-Jet Stream Technology (Agilent Technologies). A 250 mm x 4.6 mm i.d., 5 μm particle size Nucleosil 120 RP-18 column (Teknokroma) preceded by an ODS precolumn was used. Each sample (20 μL) was injected and separated isocratically by using a mobile phase consisting of water and acetonitrile, both containing 0.1% formic acid, at a flow rate of 0.4 mL/min. The Q-ToF acquisition conditions were as follows: 2 GHz, mass range between 100-1000 m/z, negative polarity, drying gas volume and temperature 8 L/min and 350ºC, sheath gas volume and temperature 11 L/min and 325ºC, nebulizer pressure 45 psi, cap voltage 3500 V, nozzle voltage 1000 V, and fragmentor voltage 75 V. Data acquisition and qualitative analysis were performed by using MassHunter Workstation Software.

2.6. Cell Culture and treatment

Human hepatic HepG2 cells were maintained in a humidified incubator containing 5 % CO_2 and 95 % air at 37 ºC. They were grown in DMEM F-12 medium from Biowhitaker (Lonza, Madrid, Spain), supplemented with 2.5 % foetal bovine serum (FBS) and 50 mg/L each of gentamicin, penicillin and streptomycin. The different concentrations of the three extracts (1, 5, 10, 20 and 40 μg/mL) were dissolved in serum-free culture medium and added to the cell plates for 20 h except in the ROS assay. In the experiments to evaluate the protective role of the compounds against an oxidative insult, cells were pre-treated with the same concentrations of the compounds for 20 h, then the medium was discarded and fresh medium containing 400 μM *t*-BOOH was added for 3 h, after which the cell cultures were processed for each assay.

2.7. Evaluation of cell viability, ROS production and antioxidant defences

Cellular damage was evaluated by lactate dehydrogenase (LDH) leakage (Alía et al., 2006). Cells were seeded (2 x 10^5 cells per plate) in 60 mm plates, grown for 20 h with the different treatments and then the cell culture medium was collected and the cells were scraped off in phosphate buffer saline (PBS). LDH leakage was estimated from the ratio between the LDH activities in the culture medium and the total activity, culture medium plus intracellular. Cellular ROS were quantified by the dichlorofluorescin assay using a microplate reader (Alía et al., 2006). Cells were seeded in 24-well plates (2×10^5 cells per well) Multiwell plates were seeded as previously referred and measured in a fluorescent microplate reader at excitation wavelength of 485 nm and emission wavelength of 530 nm. GSH content was evaluated by a fluorometric assay (Alía et al., 2006). The method takes advantage of the reaction of GSH with *o*-phthalaldehyde at pH 8.0. Fluorescence was measured at excitation and emission wavelength of 340 nm and 460 nm respectively. Determination of GPx activity was based on the oxidation of GSH by GPx, using *t*-BOOH as a substrate, coupled to the disappearance rate of NADPH by GR (Alía et al., 2006). GR activity was determined by following the decrease in absorbance due to the oxidation of

NADPH utilized in the reduction of oxidized glutathione (Alía et al., 2006). Protein concentration in the samples was measured by the Bradford assay.

2.8. Determination of carbonyl groups and malondialdehyde (MDA)

Protein oxidation of cells was measured as carbonyl groups content in supernatants according to the method of Richert et al. (2002). Absorbance was measured at 360 nm and carbonyl content was expressed as nmol/mg protein using an extinction coefficient of 22000 nmol/L/cm. Cellular MDA was analyzed in supernatants by HPLC as its DNPH derivative (Mateos et al., 2004). An Agilent 1100 Series HPLC-DAD was used and MDA values were expressed as nmol of MDA/mg protein.

2.9. Statistics

Statistical analysis of data obtained from cell culture studies was performed as follows: prior to analysis the data were tested for homogeneity of variances by the test of Levene; for multiple comparisons, one-way ANOVA was followed by a Bonferroni test when variances were homogeneous or by Tamhane test when variances were not homogeneous. The level of significance was $P < 0.05$. A SPSS version 19.0 program was used.

3. Results

3.1. Identification and characterization of polyphenols in plant extracts

The phenolic constituents present in the three extracts of *Corema album* were monitored by DAD, MS and ESI-QTOF detection. Typical chromatograms of the three extracts of *Corema album* are shown in Figure 1. Table 1 shows the list of compounds identified along with their retention time (RT) and UV characteristics of the chromatographic peaks, $[M-H]^-$ and their corresponding fragment ions, accurate mass (acc. mass), molecular formula (MF) and mDa of error between the mass found and the accurate mass of each polyphenol. Up to 20 different phenolic compounds were detected in the extracts (A, EA and W).

3.1.1. Identification of phenolic acids

Peaks 1, 3, 4, 5 and 6 had similar UV spectra profile, with a maximum at 314–326 nm and a shoulder at 296-298 nm, typical of caffeic acid derivatives. Peaks 1, 4 and 6 presented similar mass spectra, with a $[M-H]^-$ ion at m/z 353 and ions at m/z 191, 179 and 707, corresponding to deprotonated quinic acid, caffeic acid and dimeric adduct of the caffeoylquinic acid molecule, respectively. The LC retention time and UV and MS spectra of compound 4 were identical to standard chlorogenic acid (5-*O*-caffeoylquinic acid), while 1 and 6 would correspond to chlorogenic acid isomers: 3-*O*-caffeoylquinic acid and 4-*O*-caffeoylquinic acid.

Compound 2 showed a RT, UV and MS spectra totally coincident with *p*-hydroxybenzoic acid standard. Compound 3 showed a $[M-H]^-$ ion at m/z 341 and a fragment with m/z 179. This fragmentation along with the accurate mass and molecular formula permitted its identification as caffeic acid-*O*-hexoside. Caffeic acid (peak 5) was identified by comparing its RT, UV and MS spectra with that of reference substance. Finally, compound 18 showed an UV spectrum with λmax at 278 and a shoulder at 316 nm. The accurate mass and molecular formula provided by Mass Hunter along with $[M-H]^-$ ion at m/z 293 and a fragment at m/z 193 permitted its tentative identification as 6-gingerol, although further characterization is necessary to confirm this point.

3.1.2. Identification of anthocyanins

Peaks 7, 8 and 9 had UV spectra characteristic of the anthocyanin group, with two absorption maxima at 280 nm and at 516-535 nm. The RT and UV spectra were identical to standard delphinidin 3-*O*-glucoside, cyanidin 3-*O*-glucoside and cyanidin 3-*O*-arabinoside, respectively.

3.1.3. Identification of flavonols

Six peaks (compounds 10, 11, 12, 13, 14 and 15) had UV spectra compatible with flavonols, characterized by two absorption maxima in the ranges 256-266 nm and 344-360 nm. Compound 10 had a deprotonated molecular ion at m/z 479 and a fragment at m/z 317, resulting to the loss of dehydrated hexose moiety to yield myricetin ion and confirming the presence of myricetin-3-*O*-glucoside. Compound 11 showed a MS spectrum with $[M-H]^-$ ion at m/z 463 and a fragment at m/z 301, derived from the loss of dehydrated hexose moiety to yield the quercetin ion, corresponding to quercetin-3-*O*-glucoside.

Compound 12 showed a RT, UV and MS spectra totally coincident with rutin standard. Regarding compound 13, the MS spectrum showed a $[M-H]^-$ ion at m/z 433 and a fragment at m/z 301 corresponding to quercetin after the loss of dehydrated arabinose moiety that enabled its identification as quercetin-3-*O*-arabinose.

Chromatographic peaks 14 and 15 showed identical MS spectrum, providing a deprotonated molecular ion at m/z 447 and a fragment at m/z 285 indicative of flavonol kampherol after the loss of 162 units $[M-H-162]^-$, that leaded to cautious identification as kampherol 3-*O*-galactoside and 3-*O*-glucoside, respectively.

3.1.4. Identification of flavanones

Peaks 16 and 20 showed UV spectra indicative of a flavanone structure with a λmax at 290 nm and a shoulder at 332 nm. Particularly, compound 16 showed an only ion at m/z 255 which accurate mass and molecular formula pointed to pinocembrin, as the possible identity of this peak. On the other hand, compound 20 showed $[M-H]^-$ ion at m/z 407 and a fragment at m/z 165 that permitted its identification as 6-geranylnaringenin.

3.1.5. Identification of stilbenes

Chromatographic peak 17 showed an UV spectrum with λmax at 270 and a shoulder at 304 nm. Considering the mass spectra, this compound provided the deprotonated molecular ion at m/z 255 plus two fragment ions at m/z 241 and 227, consistent with the loss of one and two methyl groups, respectively. QTOF analysis was in agreement with these results, confirming the presence of pterostilbene in *Corema album* fruit.

3.2. Quantification of phenolic compounds from *Corema album* fruit.

Contents of phenolic compounds, excluding unknown chromatographic peaks 18 and 19, were determined by HPLC-DAD and using standards and wavelength specified in materials and methods. The total content of phenolic compounds was calculated as the sum of each phenolic group. Results were expressed as mg per 100 mL of each extract. The final composition determined in the three phenolic extracts (A, EA and W) obtained from *Corema album* fruit is summarized in Table 2.

The results indicate different degree of extraction of the polyphenols groups in accordance with the polarity of the used solvent. Thus, the extract obtained with water contained the most polar compounds such as phenolic acids and anthocyanins, in detriment of the most lipophyilic ones, i.e.

flavanones and stilbenes. The extraction carried out with acetone provided the most balanced composition, recovering high amounts of all classes of polyphenols. Finally, when the extraction was developed with ethyl acetate, the extract showed high amount of flavonols and stilbenes and very low amounts of the most polar compounds. The overall comparison of the three extractants pointed out at acetone as slightly the best solvent to obtain the highest recovery rates of all classes of polyphenols from *Corema album* fruit.

3.3. Antioxidant effects of *Corema album* extracts on HepG2 in basal conditions

Cell viability remained unaltered after 20 h treatment with the three extracts at a dose as high as 40 µg/mL (Figure 2a). Treatment for 20 h with doses up to 100 µg/mL of any extract did not affect cell viability by the crystal violet assay (data not shown). Thus, it can be assumed that the range of concentrations finally selected (1-40 µg/mL) can be safely used to study the potential protective effect *in vitro* of *Corema album* extracts against a condition of oxidative stress.

Additionally, treatment of cells with 1-40 µg/mL of all three extracts significantly decreased ROS production as compared to those of control cells (Figure 2b). A dose-response was observed; thus, 20-40 µg/mL of any extract evoked the largest decrease in ROS (Figure 2b).

Treatment with increasing concentrations of extracts during 20 h provoked an increase in GSH concentration in all treatments except 10-40 µg/mL EA (Figure 3a). No significant changes in the activity of GPx were observed (Figure 3b), but treatment with 40 µg/mL EA and 20 µg/mL W evoked a significant increase in the GR activity (Figure 3c).

Biomarkers of oxidative damage to proteins and lipids were tested with only the intermediate dose of 10 µg/mL from the three extracts. No significant changes in the concentration of carbonyl groups were found (Figure 4a), but a significant decrease in MDA levels was observed after the treatment with 10 µg/mL of W extract (Figure 4b**)**.

3.4. Protective effects of *Corema album* extracts on HepG2 in a condition of oxidative stress

Treatment with *t*-BOOH for 3 hours evoked a prominent cell death of around 40 % (Figure 5a). Pre-treatment for 20 h with 1-40 µg/mL of all three extracts significantly reduced cell damage induced by *t*-BOOH, limiting cell death to values that were below 10 % in A- and W-treated HepG2 and around 15 % in AE-treated cells (Figure 5a).

Cells treated with *t*-BOOH showed a significant 2.5-fold increase in ROS generation after 2 h as compared to non-stressed controls (Figure 5b). Pre-treatment with 40 µg/mL A for 20 h slightly but significantly decreased ROS generation induced by *t*-BOOH, yet a remarkable ROS reducing effect was observed with 1-40 µg/mL EA and W (Figure 5b).

Addition of *t*-BOOH to cells for 3 h evoked a dramatic depletion of the GSH concentration to 40 % of control values (Figure 6a); this diminution was partly overcome by pre-treatment with all doses of EA extract and 1-20 µg/mL of A and W, and completely surmounted by treatment with 40 µg/mL of A and W (Figure 6a). Treatment with *t*-BOOH for 3 h induced a 2-fold increase in the activity of GPx and GR (Figure 6b,c) as a defence response against the oxidative insult. Pre-treatment with all doses of the three extracts completely reversed the chemically induced increase in GPx and GR (Figure 6b,c).

Treatment with *t*-BOOH during 3 h evoked a 4-fold increase in the concentration of carbonyl groups, indicating permanent oxidative damage to cell proteins (Figure 7a). Pre-treatment with the three

extracts evoked a partial (EA) or complete (A and W) recovery of the carbonyl concentration (Figure 7a). Treatment with *t*-BOOH during 3 h also evoked a 2-fold increase in the concentration of MDA, indicating permanent oxidative damage to cell lipids (Figure 7b). Pre-treatment with 10 µg/mL of A, EA and W extracts for 20 h greatly prevented the MDA increase induced by *t*-BOOH. Indeed, a complete reduction of the chemically-induced MDA was observed in cells that had previously been treated with 10 µg/mL of EA and W (Figure 7b).

4. Discussion

In this study, we analyzed the phenolic composition of three different extracts obtained from the berry of the plant *Corema album* and investigated the chemo-protective effect of the extracts against induced oxidative stress and the different defence mechanisms involved. The results proclaim hydroxycinnamic acids as the major phenolic fraction in the three plant extracts and demonstrate that pre-treatment of hepatic cells with any of the extracts grant significant protection against an oxidative challenge chemically induced.

Chemical characterization of the extracts showed a remarkable similarity in the total amount of phenolic compounds, 1.4-1.5 g/100 g extract. Around 80% and 90% of the phenolic fraction of extracts A and W, respectively, is composed by hydroxycinnamic acids that almost double the amount of these compounds in EA extract. However, the latter fraction compensated with a significantly higher amount of flavonoids (particularly flavanones), phenolic acids and stilbenes, although hydroxycinnamic acids still amounted to 50% of the total phenolics. Between 1.2-1.3% of hydroxycinnamic acids is a relatively high amount compared to other fruits and vegetables but remains far from roasted coffee beans, which comprise a higher concentration of 14% of chlorogenic acids and related compounds (Farah & Donangelo, 2006). All three extracts were poor in anthocyanins. Therefore, most of the biological effects of the extracts should be endorsed to the group of hydroxycinnamic acids, in particular, 5-*O*-caffeoylquinic acid or chlorogenic acid.

Biological activities of natural hydroxycinnamic acids include inhibiting tumour cell proliferation (Caillet et al., 2012; Janicke et al., 2011) and anti-inflammatory activity (Kim et al., 2012; Nagasaka et al., 2007). Acute ingestion of yerba mate infusion rich in hydroxycinnamic acids inhibits plasma and lipoprotein oxidation in humans (Da Silva et al., 2008). Caffeoylquinic acid has shown liver-protective activity in experimental liver injury models (Basnet et al., 1996) and its administration to rats improved glucose and lipid homeostasis and overall antioxidant status (Jurgoński et al., 2012). Besides, chlorogenic acid up-regulated cellular antioxidant enzymes in human adenocarcinomic alveolar cells (Feng et al., 2005), and a significant protective effect of chlorogenic acid against an induced oxidative stress has been reported in PC12 cells (Pavlica & Gebhardt, 2005). Finally, chlorogenic acid enhanced the intrinsic cellular tolerance against oxidative insults in HepG2 cells both by activating survival/proliferation pathways and increasing antioxidant potential (Granado-Serrano et al., 2007).

All these properties of chlorogenic acid make *Corema album* phenolic extracts interesting candidates for cellular chemo-protection, and, to our knowledge, there is no previous data on cell culture-based study testing the antioxidant effects of *Corema album* extracts. Although natural phenolics may have potent antioxidant effects *in vitro* and *in vivo*, elevated doses of dietary antioxidants may also act as

pro-oxidants in cell culture systems and provoke cellular damage (Azam et al., 2004). Consequently, the selection of the tentative range of doses used was based on both a literature search and our previous studies with other phenolic extracts. Thus, authors have reported human blood concentrations up to 25 µM of chlorogenic acid after ingestion of a green coffee extract (Farah et al., 2008), blood levels up to 10 µM in humans after coffee consumption (Monteiro et al., 2007) and up to 1 µM of all hydroxycinnamic acids and their metabolites in plasma after ingestion of coffee (Stalmach et al., 2009). In a previous work we have shown a chemo-protective effect on HepG2 cells with 0.5-50 µg/mL of cocoa phenolic extract (Martín et al., 2008; Martín et al., 2010a). Therefore, in order to evaluate the effect of *Corema album* extracts, the concentration range selected, 1-40 µg/mL, is not far from realistic. In terms of chlorogenic acid, this range is equivalent to 2.8-113 µM. Cell integrity, redox status and oxidative stress biomarkers were primarily determined under these conditions, then, once ensured that the range of concentrations is secure, the response of *Corema album* extracts-conditioned cells against an oxidative challenge was tested.

As expected, treatment of cells with 1-40 µg/mL of the three extracts for 20 h produced no significant cell damage and evoked a dose-response reduction in the cellular ROS production, in agreement with previous reports indicating that plant hydroxycinnamic compounds are effective scavengers of oxygen radicals in cell cultures (Feng et al., 2005; Pavlica and Gebhardt, 2005). The same treatment with *Corema album* extracts evoked a substantial increase in GSH, reflecting a reduced intracellular oxidation which could be expected to prepare the cell against a potential oxidative insult. In line with this result, treatment of HepG2 cells with chlorogenic acid (Granado-Serrano et al., 2007) also resulted in an increase in steady-state GSH concentrations.

The presence of glutathione-dependent enzymes is essential to prevent the cytotoxicity of ROS. An increase in the steady-state activity of antioxidant enzymes with a cocoa phenolic extract (Martín et al., 2010a) and olive oil phenolic hydroxytyrosol (Martín et al., 2010b) has been reported. In accordance with those results, the present study shows a significant increase in GR activity in HepG2 cells treated with high concentrations of EA and W extracts for 20 h. This outcome pointed out that these treated cells were in better conditions to face the increasing generation of ROS induced by the potent pro-oxidant *t*-BOOH. Additionally, W extract significantly reduced MDA, a three-carbon compound formed by scission of peroxidized polyunsaturated fatty acids. Since MDA has been found elevated in various diseases thought to be related to free radical damage, it has been widely used as an index of lipid peroxidation in biological and medical sciences (Breusing et al., 2010).

Therefore, cells treated with the extracts seem to be in favourable conditions to face an oxidative challenge. Treatment of HepG2 cells with the strong prooxidant *t*-BOOH is an excellent model of oxidative stress in cell culture systems (Alía et al., 2006; Lima et al., 2006; Martín et al., 2008; Martín et al., 2010a). Thus, 400 µM *t*-BOOH significantly enhanced cell damage and ROS generation in HepG2, and pre-treatment of HepG2 cultures with 1-40 µg/mL of the three *Corema album* extracts greatly prevented cell damage and slightly but significantly reduced ROS. These results suggest that the ROS generated during the period of oxidative stress were more efficiently quenched in cells pre-treated with extracts, which could be a first explanation for the reduced cell damage.

Addition of *t*-BOOH to cells evoked a remarkable depletion of GSH which was significantly overcome by pre-treatment of the cells with any of the three *Corema album* extracts. These results implied that increased levels of GSH in the extract-treated cells before exposure to the oxidative challenge greatly helped to prevent the dramatic depletion of the intracellular GSH stock. Maintaining GSH concentration above a critical threshold while facing a stressful situation represents a decisive advantage for cell survival.

Induction of GPx and GR are critical mechanisms of the cell defence against oxidative insults and plays a major role to overcome ROS production in the presence of *t*-BOOH (Alía et al., 2006; Martín et al., 2008; Martín et al., 2010a,b). However, a rapid return of the antioxidant enzyme activities to basal values once the challenge has been surmounted will place the cell in a favorable condition to deal with a new insult. In this study, pre-treatment of cells with *Corema album* extracts managed to prevent the long-lasting increase in the activities of GPx and GR induced by oxidative stress. This ensured that the cells were in optimum conditions to withstand further oxidative challenges. In concert, we have previously reported that a cocoa phenolic extract averted cell damage by preventing the permanently increased activities of GPx and GR induced by *t*-BOOH (Martín et al., 2008; Martín et al., 2010a).

The significant increase in the cellular concentration of carbonyl groups and MDA during oxidative stress induced by *t*-BOOH indicated extensive damage to cellular proteins and lipids. Pre-treatment of HepG2 with 10 µg/mL of the three extracts significantly decreased both biomarkers demonstrating a reduced degree of protein and lipid oxidation in response to the stressful situation. This chemo-protective effect on oxidative markers has also been reported with other plant phenolic extracts (Martín et al., 2008; Martín et al., 2010a) and pure natural bioactive compounds (Alía et al., 2006; Martín et al., 2010b). Therefore, as evidenced by the results of ROS production, GSH concentration and antioxidant enzymes activity, the rapid recovery of the redox homeostasis evoked by the pre-treatment with *Corema album* extracts will limit protein and lipid degradation and will ensure reduced cell damage.

Despite the different composition of phenolic compounds in extracts A and W (around 85% hydroxycinnamics and 15% of rest of polyphenols) and EA (60% hydroxycinnamics, 26% flavonoids and 12% of stilbenes), the chemo-protective efficiency of the three fractions was quite similar. Treatment with A and W reduced cell damage during oxidative stress to less than 10% whereas cells treated with EA remained slightly over 15%; further, only cells treated with A and W completely recovered GSH. However, EA extract evoked in stressed cells a stronger ROS and MDA reduction than extract A and, overall, had a comparable effect on the rest of parameters. This suggests that the significant amount of flavonoids and stilbenes in EA extract compensates for the smaller quantity of hydroxycinnamic acids in the chemo-protective activity.

In conclusion, the results indicate that the three *Corema album* extracts tested are rich in hydroxycinnamic acids and contain different amounts of flavonoids and stilbenes. Treatment with the tested concentrations of the three extracts evoked changes in the basal redox status, such as reduced ROS and increased GSH, which place the cells in favourable conditions to face an oxidative challenge. Cells pre-treated with the extracts showed an outstanding protection against the challenge-induced damage. These results support the traditional use of *Corema album* as a medicinal plant and its presence in the diet

as an appetizer may contribute to the protection afforded by fruits, vegetables and plant-derived beverages against diseases for which oxidative stress has been implicated as a causal or contributing factor.

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Legends to figures

Figure 1.- HPLC chromatograms of phenolic compounds extracted from *Corema album* berries with acetone (A), ethyl acetate (EA) and water (W) at 280nm.

Figure 2.- Direct effect of *Corema album* extracts on cell viability and ROS generation. Panel a, LDH leakage is expressed as percent of LDH activity in the culture medium of the total activity, culture medium plus intracellular. Values are means ± SD (n=6-8). Panel b, fluorescence units corresponding to intracellular ROS production are expressed as percent of control data. Values are means (n=7-8). Means without a common letter differ, $P< 0.05$.

Figure 3.- Direct effect of *Corema album* extracts on antioxidant defences. HepG2 were treated with the noted concentrations of the extracts for 20 h. Intracellular concentration of GSH (a) and enzyme activity of GPx (b) and GR (c) are expressed as percent of control value ± SD of 4-5 different samples per condition. Different letters indicate statistically significant differences ($P < 0.05$) among different groups.

Figure 4.- Direct effect of *Corema album* extracts on oxidative stress biomarkers. HepG2 were treated with 10 μg/mL of the extracts for 20 h. Values of carbonyl groups (a) and MDA (b) are expressed as means ± SD of 4 different samples per condition. Different letters indicate statistically significant differences ($P < 0.05$) among different groups.

Figure 5.- Protective effect of *Corema album* extracts on cell viability and ROS generation. HepG2 were treated with the noted concentrations of extracts for 20 h, then the cultures were washed and 400 μM *t*-BOOH was added to all the cultures except controls for 3 h (panel a, LDH) or 2 h (panel b, ROS). Results of LDH leakage are expressed as percent of LDH activity in the culture medium of the total activity, culture medium plus intracellular and intracellular ROS production was expressed as percent of control

values. Data are means ± SD (n=6-8). Different letters indicate statistically significant differences ($P < 0.05$) among different groups.

Figure 6.- Protective effect of *Corema album* extracts on the antioxidant defences. GSH content (a) and activity of GPx (b) and GR (c) in HepG2 cells treated with the noted concentrations of the extracts for 20 h before the exposure to 400 μM *t*-BOOH during 3 h. Values are means ± SD (n=4-5). Means without a common letter differ, $P< 0.05$.

Figure 7.- Protective effect of *Corema album* extracts on oxidative stress biomarkers. HepG2 were treated with the noted concentrations of the extracts for 20 h, then the cultures were washed and 400 μM *t*-BOOH was added to all the cultures except controls for 3 h. Values of carbonyl groups (a) and MDA (b) are expressed as means ± SD of 4 different samples per condition. Different letters indicate statistically significant differences ($P < 0.05$) among different groups.

Figure 1

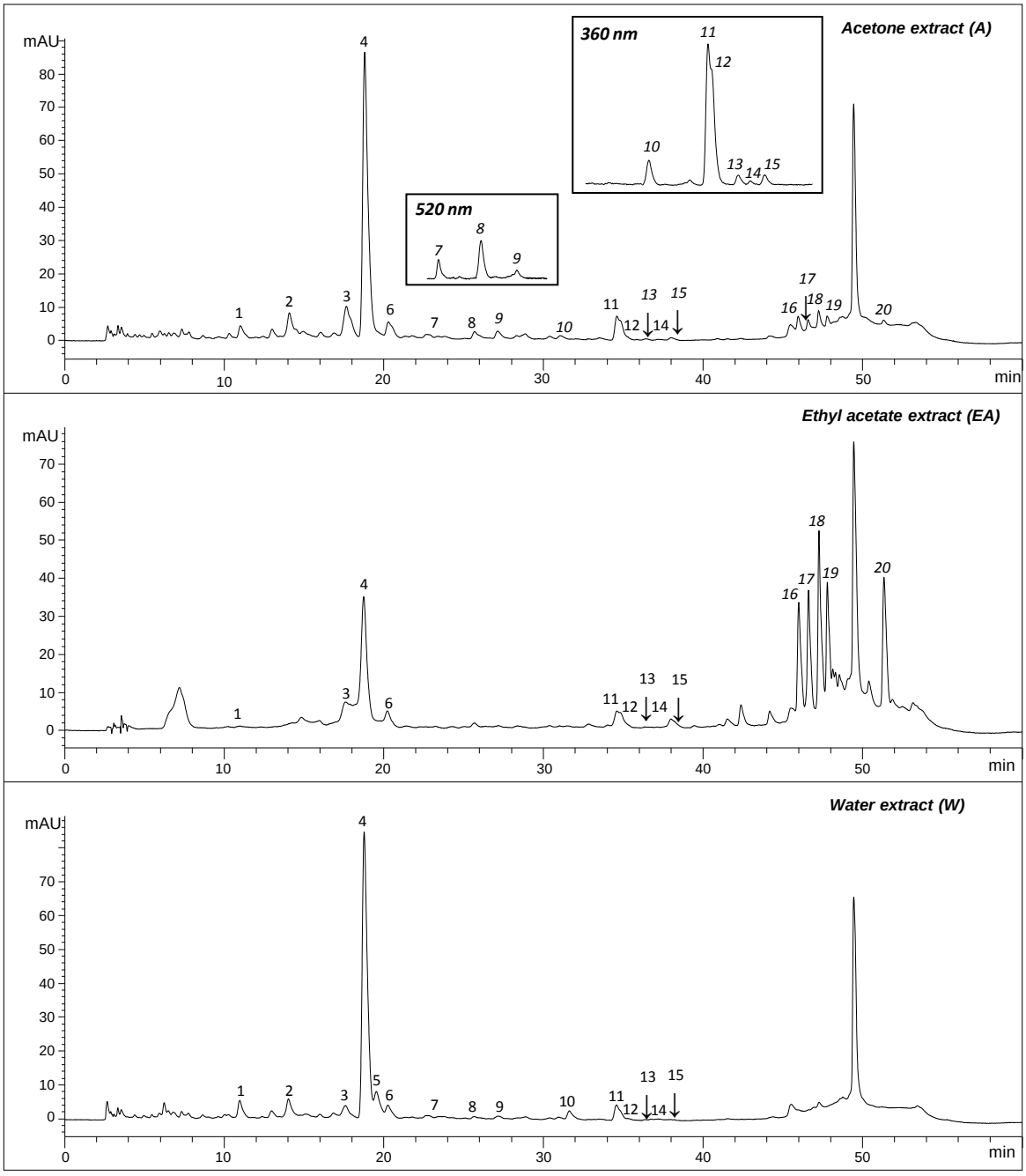

Acetone extract (A)
360 nm
520 nm
Ethyl acetate extract (EA)
Water extract (W)
mAU
min

Figure 2

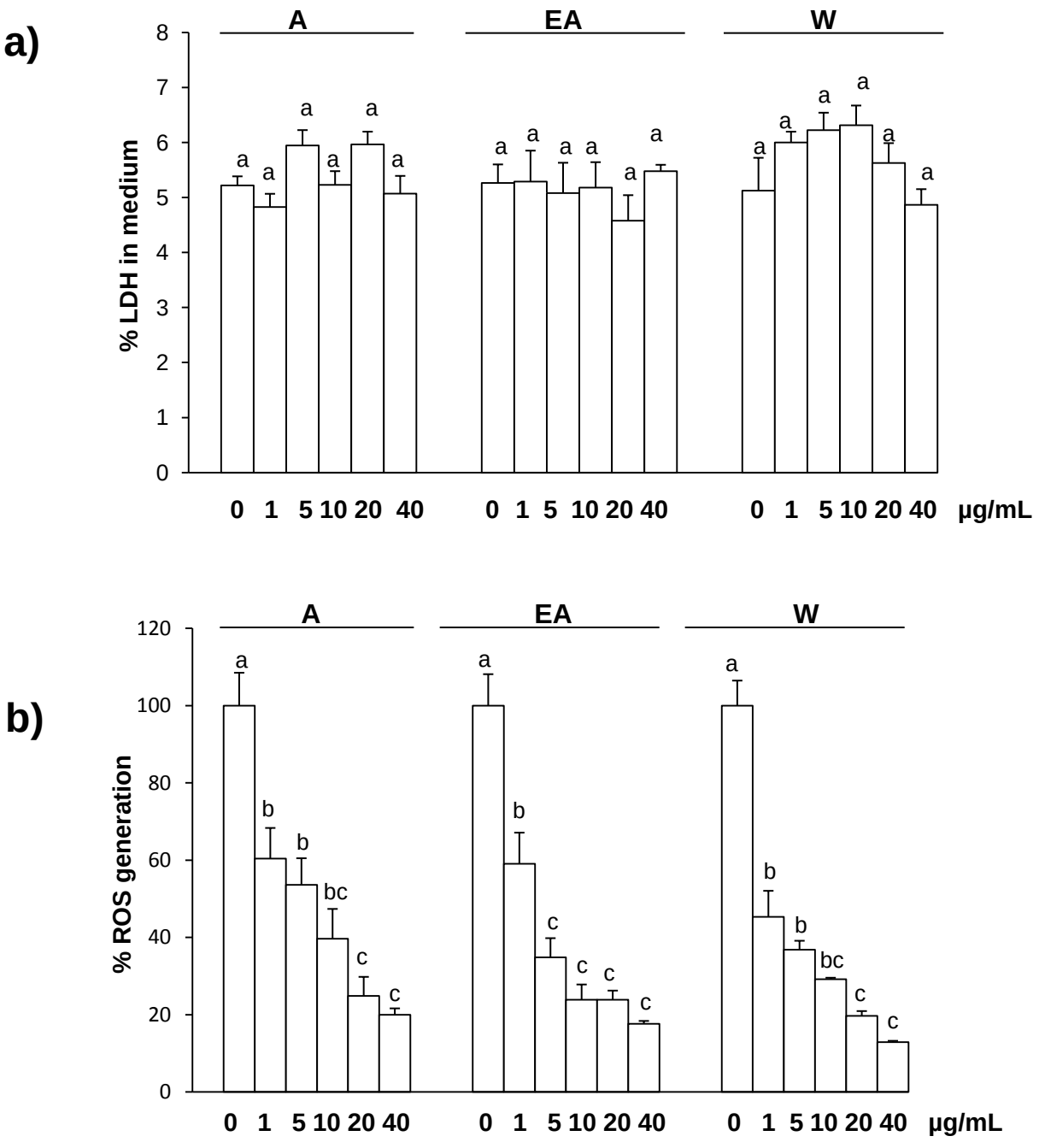

a)
A
EA
W
% LDH in medium
0 1 2 3 4 5 6 7 8
0 1 5 10 20 40
µg/mL
b)
% ROS generation
0 20 40 60 80 100 120
a b b bc c c
a b c c c c
a b b bc c c

Figure 3

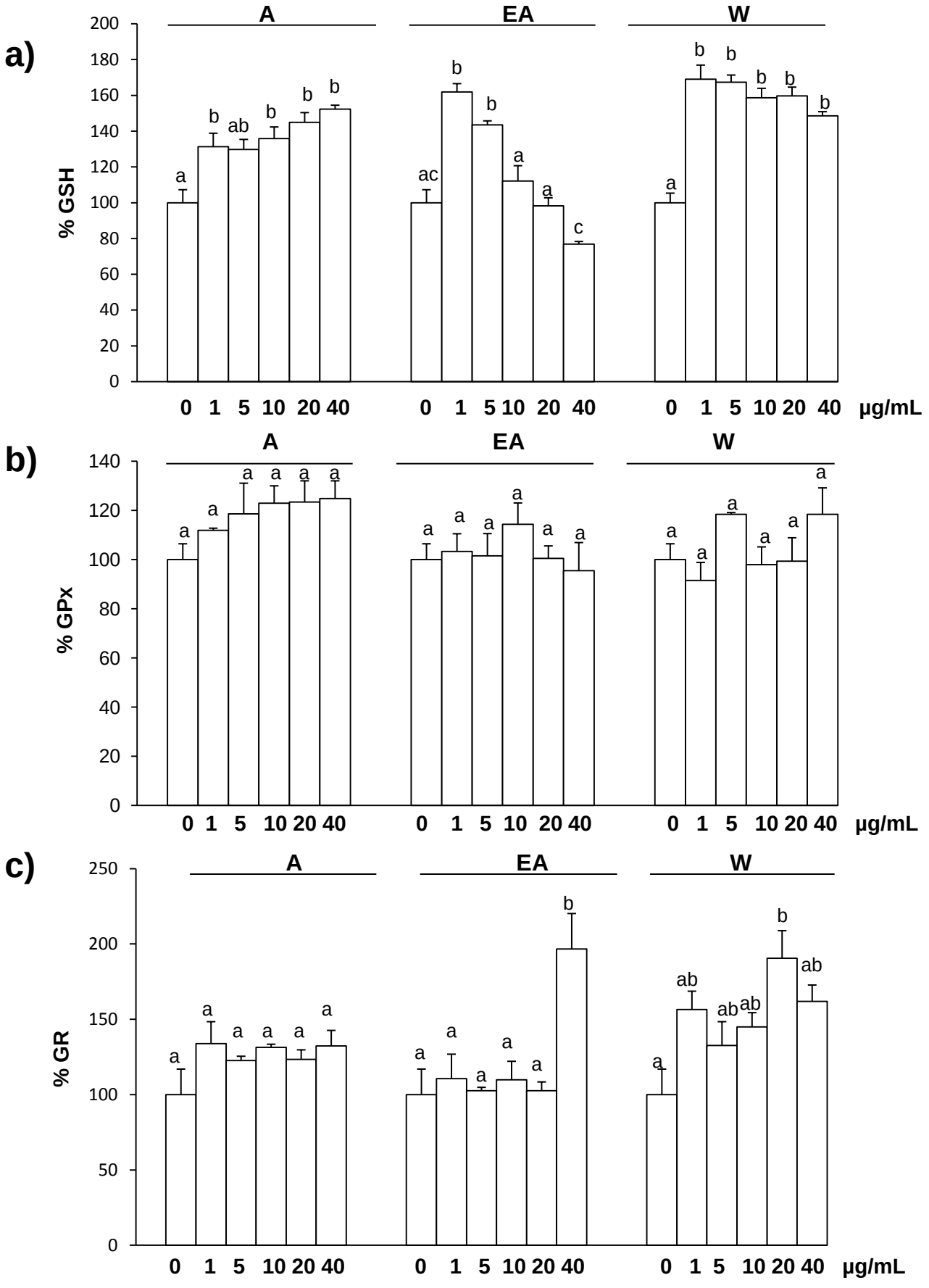
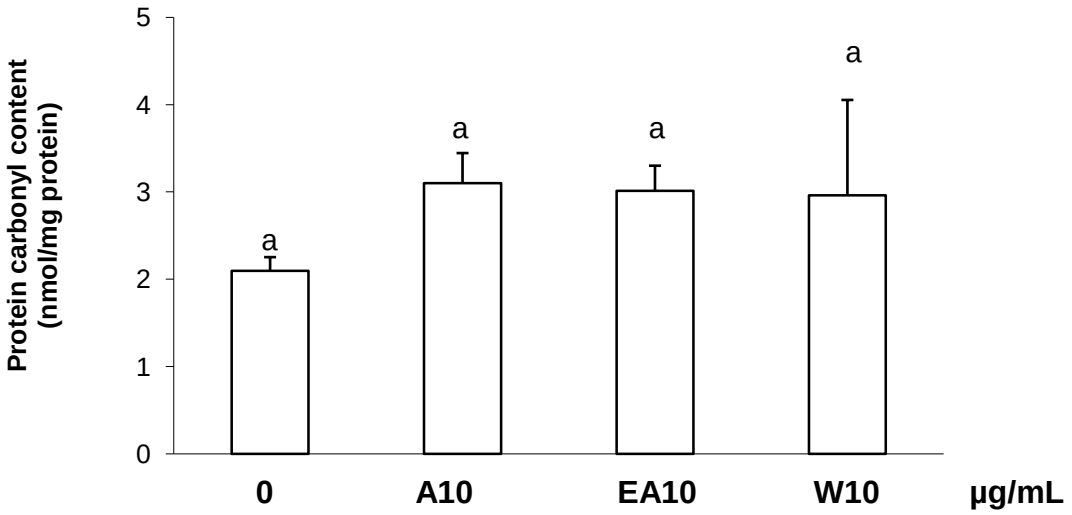

a)
A
EA
W
% GSH
200
180
160
140
120
100
80
60
40
20
0
a b ab b b b
ac b b a a c
a b b b b b
0 1 5 10 20 40
µg/mL
b)
% GPx
140
120
100
80
60
40
20
0
a a a a a a
a a a a a a
a a a a a a
c)
% GR
250
200
150
100
50
0
a a a a a a
a a a a a b
a ab ab ab b ab

Figure 4

a)


Protein carbonyl content (nmol/mg protein)
5
4
3
2
1
0
a
a
a
a
0
A10
EA10
W10
µg/mL

b)

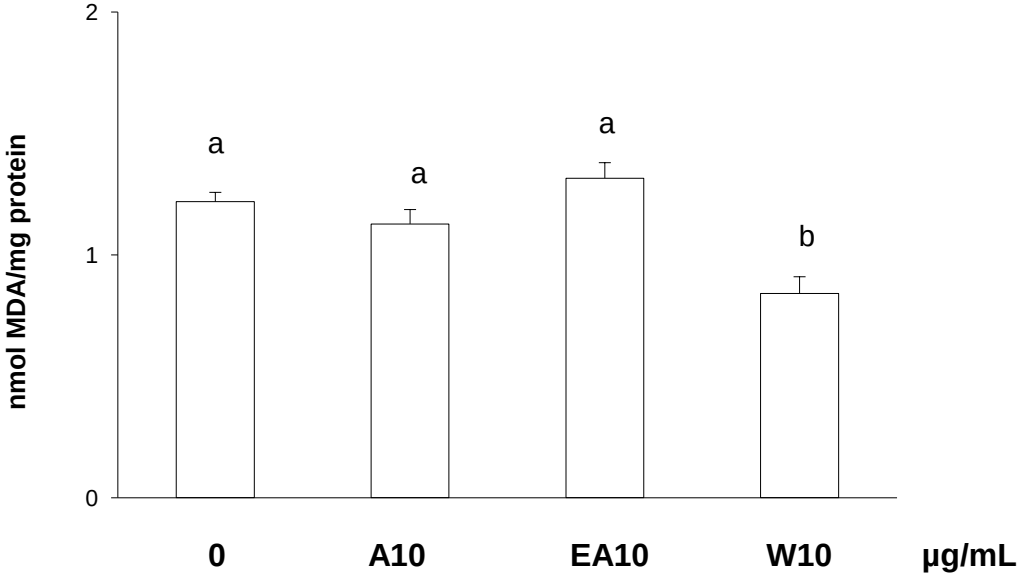

nmol MDA/mg protein
2
1
0
a
a
a
b
0
A10
EA10
W10
µg/mL

Figure 5

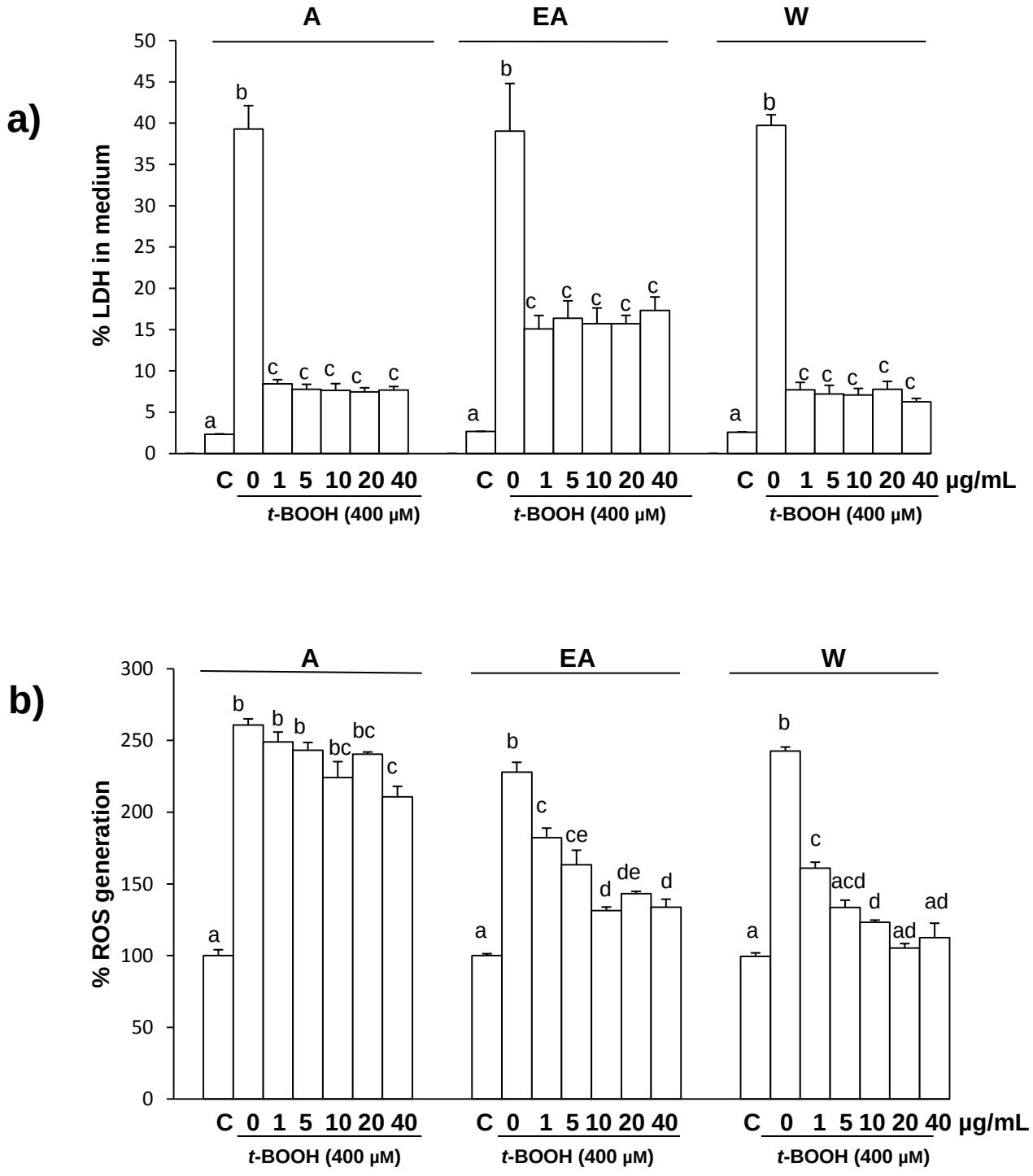

a)
A
EA
W
% LDH in medium
50
45
40
35
30
25
20
15
10
5
0
C 0 1 5 10 20 40 µg/mL
t-BOOH (400 µM)
b)
% ROS generation
300
250
200
150
100
50
0

Figure 6

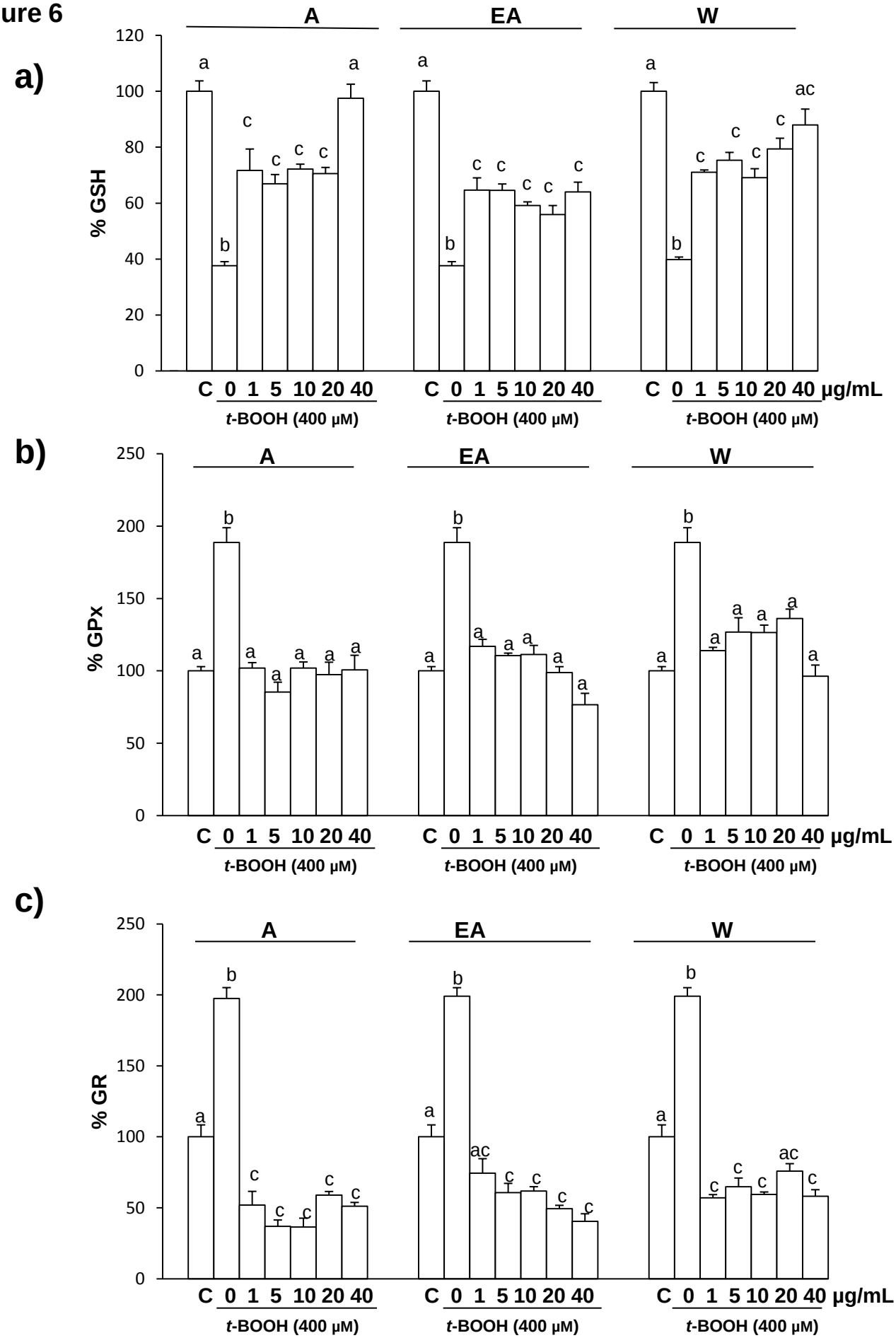
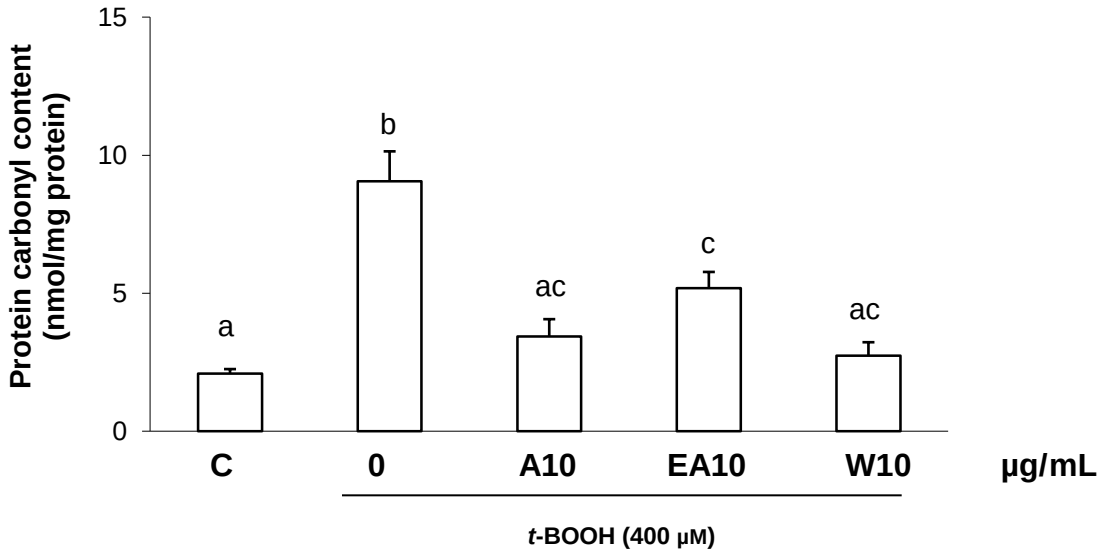
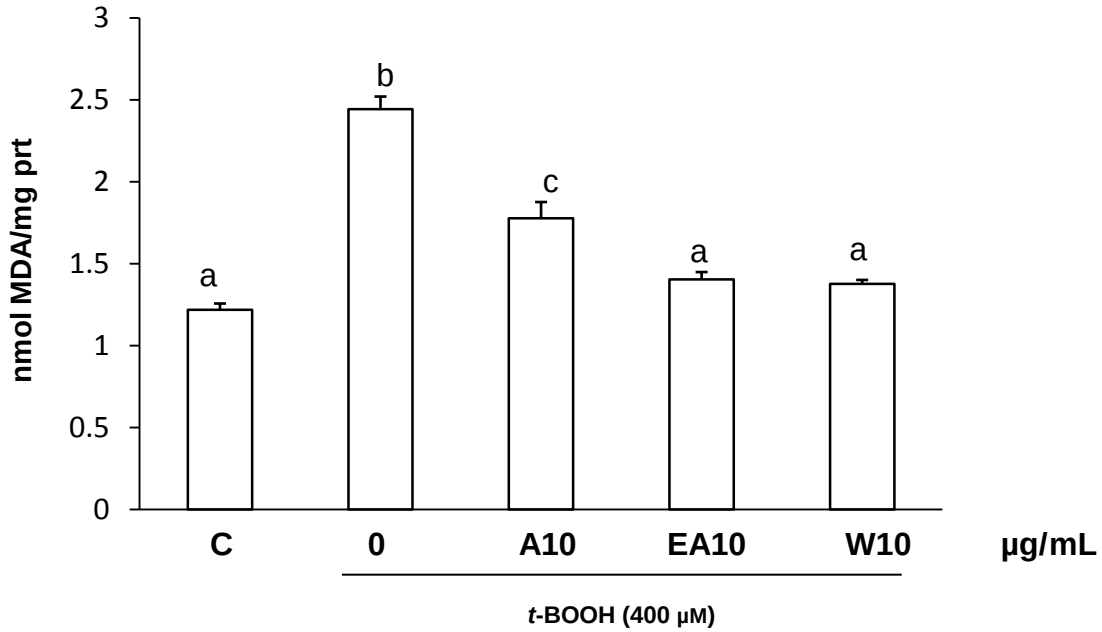

a)
A
EA
W
% GSH
120
100
80
60
40
20
0
C 0 1 5 10 20 40 µg/mL
t-BOOH (400 µM)
b)
% GPx
250
200
150
100
50
0
c)
% GR

Figure 7

a)


Protein carbonyl content (nmol/mg protein)
15
10
5
0
a
b
ac
c
ac
C
0
A10
EA10
W10
µg/mL
t-BOOH (400 µM)

b)


nmol MDA/mg prt
3
2.5
2
1.5
1
0.5
0
a
b
c
a
a
C
0
A10
EA10
W10
µg/mL
t-BOOH (400 µM)

Table

Table 1. List of compounds identified in *Corema album* fruit.

Chr. Peak	Compound	RT (min)	λmax	$[M-H]^-$	Fragment ions	Acc. mass	mDa	M.F.
1	3-*O*-Caffeoylquinic acid	11.01	326, 296sh	353	191,179,707	353.0878	-1.68	$C_{16}H_{18}O_9$
2	*p*-hydroxybenzoic acid	14.02	270	137		137.0244	-0.67	$C_7H_6O_3$
3	Caffeic acid-*O*-hexoside	17.63	316, 296sh	341	179	341.0878	-1.69	$C_{15}H_{18}O_9$
4	5-*O*-Caffeoylquinic acid	18.80	326, 296sh	353	191,179,707	353.0878	-1.78	$C_{16}H_{18}O_9$
5	Caffeic acid	19.54	324, 298sh	179	135	179.0350	-1.05	$C_9H_8O_4$
6	4-*O*-Caffeoylquinic acid	20.29	326, 296sh	353	191,179,707	353.0878	-2.33	$C_{16}H_{18}O_9$
7	Delphinidin 3-*O*-glucoside	23.51	276, 520					$C_{21}H_{20}O_{12}$
8	Cyanidin 3-*O*-glucoside	25.64	282, 510					$C_{21}H_{20}O_{11}$
9	Cyanidin 3-*O*-arabinoside	28.35	280, 512					$C_{20}H_{18}O_{10}$
10	Myricetin-3-*O*-glucoside	31.06	258, 358	479	317	479.0831	-0.95	$C_{21}H_{20}O_{13}$
11	Quercetin-3-O-glu coside	34.60	256, 354	463	301	463.0882	-2.25	$C_{21}H_{20}O_{12}$
12	Rutin	35.10	256, 354	609		609.1461	-0.21	$C_{27}H_{30}O_{16}$
13	Quercetin-3-*O*-arabinoside	36.41	256, 360	433	301	433.0776	-1.09	$C_{20}H_{18}O_{11}$
14	Kampherol-3-*O*-galactoside	37.11	266, 346	447	285	447.0933	-0.30	$C_{21}H_{20}O_{11}$
15	Kampherol-3-*O*-glucoside	38.01	266, 344	447	285	447.0933	-1.02	$C_{21}H_{20}O_{11}$
16	Pinocembrin	45.48	290, 332sh	255		255.0663	-1.46	$C_{15}H_{12}O_4$
17	Pterostilbene	45.96	270, 304sh	255	241, 227	255.1027	-0.62	$C_{16}H_{16}O_3$
18	Unknown	46.59	278, 316sh	293	193	293.1758	-1.40	$C_{17}H_{26}O_4$
19	Unknown	47.24	335	265				
20	6-Geranylnaringenin	51.33	290, 332sh	407	165	407.1864	-2.76	$C_{25}H_{28}O_5$

Chr. Peak: chromatographic peak; RT (min): retention time (min); Acc. Mass: accurate mass; M.F.: molecular formula; mDa: millidalton of error between the mass found and the accurate mass of each polyphenol.

Table

Table 2. Content of phenolic compounds and flavonoids in the three extracts (A, EA and W) obtained from *Corema album* fruit, expressed in mg per 100 g of extract. Results are expressed in mean ± SD of three determinations.

	A **mg/100g extract**	**EA** **mg/100g extract**	**W** **mg/100g extract**
PHENOLIC ACIDS			
Hydroxybenzoic acids (280nm)			
p-hydroxybenzoic acid	64.66 ± 0.02	0.00 ± 0.00	38.69 ± 0.02
Hydroxycinnamic acids (320nm)			
3-*O*-Caffeoylquinic acid	35.35 ± 0.02	8.13 ± 0.04	50.63 ± 0.02
Caffeic acid-*O*-hexoside	83.94 ± 0.02	70.54 ± 0.04	34.73 ± 0.03
5-*O*-Caffeoylquinic acid	1041.08 ± 0.01	599.70 ± 0.02	1042.11 ± 0.01
Caffeic acid	0.00 ± 0.00	0.00 ± 0.00	93.08 ± 0.01
4-*O*-Caffeoylquinic acid	44.46 ± 0.03	57.86 ± 0.03	38.35 ± 0.02
Total Phenolic acids	1269.49 ± 0.04	736.23 ± 0.04	1297.59 ± 0.03
ANTHOCYANINS (520nm)			
Delphinidin 3-*O*-glucoside	0.52 ± 0.04	0.00 ± 0.00	0.88 ± 0.05
Cyanidin 3-*O*-glucoside	1.13 ± 0.04	0.00 ± 0.00	1.01 ± 0.04
Cyanidin 3-*O*-arabinoside	0.26 ± 0.04	0.00 ± 0.00	0.52 ± 0.04
Total Anthocyanins	1.91 ± 0.05	0.00 ± 0.00	2.40 ± 0.05
FLAVONOLS (360nm)			
Myricetin-3-*O*-glucoside	16.43 ± 0.03	10.69 ± 0.03	11.75 ± 0.03
Quercetin 3-*O*-glucoside	110.99 ± 0.02	84.64 ± 0.04	55.69 ± 0.03
Rutin	18.31 ± 0.02	15.96 ± 0.02	14.91 ± 0.03
Quercetin 3-*O*-arabinoside	6.90 ± 0.03	5.37 ± 0.05	4.43 ± 0.04
Kampherol-3-*O*-galactoside	2.44 ± 0.04	2.80 ± 0.05	4.79 ± 0.04
Kampherol 3-*O*-glucoside	6.65 ± 0.03	3.52 ± 0.05	2.35 ± 0.04
Total flavonols	161.71 ± 0.05	122.98 ± 0.05	93.92 ± 0.05
FLAVANONES (280nm)			
Pinocembrin	15.97 ± 0.02	93.98 ± 0.02	0.00 ± 0.00
6-geranylnaringenin	7.83 ± 0.03	107.50 ± 0.02	0.00 ± 0.00
Total flavanones	23.80 ± 0.03	201.48 ± 0.02	0.00 ± 0.00
STILBENES (280nm)			
Pterostilbene	15.92 ± 0.03	147.57± 0.02	0.00 ± 0.00
Total stilbenes	15.92 ± 0.03	147.57 ± 0.02	0.00 ± 0.00
TOTAL PHENOLIC COMPOUNDS	**1472.83 ± 0.05**	**1208.27 ± 0.05**	**1393.91 ± 0.06**