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Effects of dietary extra virgin olive oil and its fractions on antioxidant status and DNA damage in the heart of rats co-exposed to aluminum and acrylamide†

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Oxidative stress generated by an excessive production of free radicals has been linked to the development of several health problems such as cardiovascular diseases. We investigated the protective efficacy of Extra Virgin Olive Oil (EVOO) and its lipophilic fraction (OOLF) and hydrophilic fraction (OOHF) against the cardiotoxicity and DNA damage induced by co-exposure to aluminum ($AlCl_3$) and acrylamide (ACR). Rats were divided into eight groups of six each: controls, $AlCl_3$ (50 mg per kg body weight) administered *via* drinking water and ACR (20 mg per kg body weight) given by gavage, combined group plus EVOO (300 μl); combined group plus the hydrophilic fraction (1 ml); combined group plus the lipophilic fraction (300 μl); extra virgin olive oil (EVOO) and its fractions were administered daily by gavage for 21 days. Three other groups, considered as positive controls, received either EVOO, OOLF or OOLH. Exposure of rats to both $AlCl_3$ and ACR provoked oxidative stress objectified by an increase in MDA, AOPP and a decrease in GSH, NPSH and vitamin C levels. The activities of CAT, GPx and SOD were also decreased. EVOO and its OOLF fraction exhibited a pronounced enhancement of antioxidant status while a partial recovery in the antioxidant status was obtained with the OOHF fraction. Plasma LDH and CK activities, TC, LDL-C levels, TC/HDL-C and LDL-C/HDL-C ratios were increased, while HDL-C and TG decreased in rats treated with both $AlCl_3$ and ACR. Co-administration of EVOO, OOLF or OOHF to treated rats restored cardiac biomarkers and lipid profile to near-normal values. Histological studies and DNA damage confirmed the biochemical parameters and the beneficial role of EVOO and its two fractions. Our results suggest that extra virgin olive oil and its two fractions can decrease the frequency of cardiac complications and genotoxicity.

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Introduction

Aluminum (Al) is the most common metal on the earth's crust and constitutes 8.13% of it. It is considered as a pro-oxidant promoting biological oxidation both *in vitro* and *in vivo*.[1] This metal disrupts the pro-oxidant/antioxidant balance in tissues leading to biochemical and physiological dysfunction due to excessive reactive oxygen species (ROS) generation. Al accumulates in all tissues of mammals, preferentially in kidneys, liver, heart, bones and brain.[2] The mechanism of Al-induced toxicity may be attributed to the potentiation of Fe^{2+} to Fe^{3+} causing oxidative damage.[3] The sources of Al are especially corn, yellow cheese, salt, herbs, spices, tea, cosmetics, cookware and containers. It is highly ubiquitous in the environment and is often found as a component of materials used in manufacturing (clays and glasses), pharmacological agents, antiperspirants and cooking.[4] The increasing use in preparation and storage of food in Al vessels, cans and foils may increase food Al content, particularly of those which are salty, acidic or alkaline.[5] The most usual Al exposure for the general population is through the diet, mainly from dietary additives used as rising and anti-caking agents, dyes and for pH adjusting.[5–8] Acryl-

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amide, another dietary contaminant, was found in various foods such as chips, crisps and bread but also biscuits.[9] Its mechanism of formation involves Maillard-type reactions which can be aggravated by aluminum transferred from cookware and utensils into the food. The formation of acrylamide (ACR) is particularly associated with high temperature cooking processes for certain carbohydrate-rich foods, especially when asparagine reacts with sugars.[10] Moreover, it is well-established that processing conditions, such as time, temperature and food matrix, dramatically influence acrylamide formation.[11] The latter is widely used in industrial fields to produce polyacrylamide and is extensively applied as a flocculant for drinking water clarification and for municipal waste water treatment.[12] There are two major biotransformation routes for acrylamide: direct conjugation with glutathione or the formation of glycidamide by oxygenation *via* cytochrome P450 (CYP2E1).[13] Once ingested by humans, acrylamide and its epoxide metabolite, glycidamide bind to either hemoglobin or DNA to form adducts and can interact with other proteins at the cellular level.[14] It is known that ACR can be cytotoxic and genotoxic by decreasing the oxidative defense system in the cells,[15] as well as, by releasing the reactive oxygen species (ROS).[16] Oxidative stress may play a crucial role in cardiac and vascular abnormalities in different types of cardiovascular diseases.[17]

The pro-oxidant/antioxidant balance plays an important role in the protection of the heart to allow normal cardiac contractile performance. Extra Virgin Olive Oil (EVOO), one of the most important food products in Mediterranean countries, can reduce the risk of coronary heart disease (CHD) by decreasing levels of artery-clogging lipids in the blood.[18] The protective role of EVOO is the result of its specific composition including high levels of phenolic compounds, α-tocopherol, monounsaturated fatty acids (MUFA) and other minor compounds.[19] The dietary MUFA health effects were attributed to a decreased endothelial activation,[20] and LDL susceptibility to oxidation.[21] Furthermore, phenolic compounds act as antioxidants against reactive oxygen species through various mechanisms, preventing first chain initiation by scavenging initiating radicals, metal chelating, decreasing localised oxygen concentration, and decomposing peroxides.[22] *In vivo* and *in vitro* studies have suggested that phenolic hydroxytyrosol (HTy) and oleuropein compounds in EVOO are the effective antioxidants through the inhibition of lipid peroxidation and by scavenging free radicals.[23] The main antioxidants of EVOO are carotenoids and phenolic compounds, which are both lipophilic and hydrophilic. The lipophilics include tocopherols, while the hydrophilics include flavonoids, phenolic alcohols and acids, secoiridoids and their metabolites.

Human beings are simultaneously exposed to several chemicals which act jointly to induce mixture effects. Thus, the present study was planned to elucidate the interaction of aluminium and acrylamide and whether extra virgin olive oil and its lipophilic and hydrophilic fractions were able to prevent oxidative stress and DNA damage in the hearts of rats co-exposed to $AlCl_3$ and ACR.

Materials and methods

Chemicals

Acrylamide (ACR), aluminium chloride ($AlCl_3$), glutathione (oxidized and reduced), nicotinamide adenine dinucleotide phosphate reduced form (NADPH), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and thiobarbituric acid (TBA) were purchased from Sigma (St. Louis, MO, USA). All other chemicals, of analytical grades, were purchased from standard commercial suppliers.

Oil samples and membrane analysis

Biologic extra virgin olive oil (EVOO) samples were obtained from a Chetoui variety cultivar grown in the North of Tunisia. The hydrophilic fraction (OOHF) was extracted from EVOO by the method of Montedoro *et al.*[24] using water instead of methanol to avoid its toxic effect in rats. Briefly, 10 g of EVOO was homogenized with 10 mL of water by using a mixer (Ultra-Turrax T25 [IKA Labortechnik, Janke & Kunkel, Staufen, Germany]; 15 000*g* per min) and centrifuged at 5000*g* for 10 min. The extraction was performed two times. The lipophilic fraction (OOLF) was obtained from EVOO as follows. The OOLF was filtered through a hydrophobic composite ceramic membrane prepared totally from the phosphate industry subproduct material. The cross flow experiments were conducted using a pilot plant made in our laboratory using a single channel tubular membrane at a temperature of 25 °C. The operating pressure was applied using a nitrogen gas source. The total active area of the membrane is 19.6 cm^2. Before experiments, the elaborated membrane was conditioned by immersion in pure deionized water for at least 24 h, and then the membrane permeability was determined (Table 1). This material was previously used as a membrane support.[25]

Different constituents of Extra Virgin olive oil and its fractions were analyzed:

– Fatty acids were converted into fatty acid methyl esters (FAMEs) prepared by dissolving 0.1 g of EVOO or OOLF in methanol and incubated for 1 hour. Individual FAMEs were separated and quantified by gas chromatography using a model 5890 series II instrument (Hewlett-Packard Ca, Palo Alto, Calif., USA) equipped with a flame ionization detector and a fused silica capillary column HP – INNOWAX (30 m

Table 1 The principal characteristics of the ceramic membrane

Characteristics	Contact angle (°)	Configuration	Surface area (cm^2)	Permeability ($L/h\ m^{-2}$ bar)
Values	160	Tubular	19.6	24

length × 0.25 mm i.d. and 0.25 μm of film thickness). The temperature was programmed to increase from 170 to 270 °C at a rate of 5 °C min^{-1}. Nitrogen ultra was used as a carrier gas. The results were expressed as the relative area percent of the total FAMEs.[26]

– Carotenoids and chlorophylls (mg per kg oil) were determined at 470 and 670 nm, respectively, in cyclohexane using the specific extinction values according to the method of Minguez-Mosquera's *et al.*[27]

– Phenolic compounds were extracted, estimated colorimetrically at 765 nm using the Folin–Ciocalteu reagent, and expressed as hydroxytyrosol equivalents as reported by Montedoro *et al.*[24]

– α-Tocopherol was evaluated according to the method of Gimeno *et al.* (2000).[28] Each oil sample was diluted with *n*-hexane (1 : 10), the mixture was vortexed and 200 μl were transferred to a test tube containing 600 μl of methanol and 200 μl of an internal standard (300 μg ml^{-1}). HPLC separation was carried out on a Hewlett-Packard system (Waldbronn, Germany) equipped with a HP-1100 pump, a Rheodyne model 7725 injector (Cotati, CA, USA, loop volume 20 μl), a HP-1200 M multi-array detector and a Supelcosil ODS-2 column (150 × 4.5 mm id., film thickness 5 μm).

Animals and treatment

Female Wistar rats, weighing 160 ± 10 g, were obtained from the Central Pharmacy (SIPHAT, Tunisia). They were housed at ambient temperature (22 ± 2 °C) in a 12 h light/dark cycle and a minimum relative humidity of 40%. Food (SNA, Sfax, Tunisia) and water were available *ad libitum*. One week after acclimatization to laboratory conditions, the rats were randomly divided into eight groups of six each.

Group A: a control group where rats received daily distilled water and a standard diet.

Group B: rats received daily acrylamide (ACR) by gavage at a dose of 20 mg per kg bw and aluminium chloride ($AlCl_3$) *via* drinking water at a dose of 50 mg per kg bw.

Group B/EVOO: rats received daily ACR and $AlCl_3$ at the same doses and by the same ways as group B. They also received daily by gavage 300 μL of EVOO.

Group B/OOLF: co-exposed rats to $AlCl_3$ and ACR received 300 μL of lipophilic fraction by gavage.

Group B/OOHF: co-exposed rats to $AlCl_3$ and ACR received 1 ml of hydrophilic fraction by gavage. Three other groups, considered as positive controls, received either EVOO, OOLF or OOLH.

Water and food intake by rats and their body weights were recorded daily. At the end of the experimental period (21 days), all rats were euthanized by cervical decapitation to avoid stress. The trunk blood was collected into heparinized tubes and centrifuged at 2200*g* for 10 min. Plasma samples were drawn and stored at −80 °C until analysis. Hearts were dissected out, cleaned and weighed. Some portions of the hearts were rinsed, homogenized in an appropriate buffer (pH = 7.4) and centrifuged. The resulting supernatants were maintained at −80 °C for biochemical assays. Other portions were immediately removed, cleaned, fixed in 10% buffered formalin solution and embedded in paraffin for histological studies.

The experimental procedures were carried out according to the General Guidelines on the Use of living Animals in Scientific Investigations[29] and approved by the Ethical Committee of Sciences Faculty, Sfax University.

Biochemical assays

Protein quantification. Heart protein contents were measured according to the method of Lowry *et al.*[30] using bovine serum albumin as a standard.

Malondialdehyde (MDA) measurement. The extent of lipid peroxidation by measuring thiobarbituric acid reactive substances (TBARS) in terms of malondialdehyde (MDA) formation was measured according to the method of Draper and Hadley.[31] 0.5 ml of a heart extract supernatant was mixed with 1 ml of trichloroacetic acid solution and centrifuged at 2500*g* for 10 min. The resulting supernatant (0.5 ml) and one milliliter of a solution containing 0.67% thiobarbituric acid (TBA) were incubated for 15 min at 90 °C and cooled. The mixture was measured for absorbance at 532 nm using a spectrophotometer (Jenway UV-6305, Essex, England). The malondialdehyde values were calculated using 1,1,3,3-tetraethoxypropane as a standard and expressed as nmoles of malondialdehyde per mg of protein.

AOPP levels. Advanced oxidation protein product (AOPP) levels were determined according to the method of Kayali *et al.*[32] Briefly, 0.4 ml of a heart extract supernatant was treated with 0.8 ml phosphate buffer (0.1 M; pH 7.4). After 2 min, 0.1 ml of 1.16 M potassium iodide was added to the tube followed by 0.2 ml of acetic acid. The absorbance of the reaction mixture was immediately recorded at 340 nm. The concentration of AOPP for each sample was calculated using the extinction coefficient of 261 cm^{-1} mM^{-1} and the results were expressed as nmoles per mg of protein.

Heart non-enzymatic antioxidant levels. – The GSH levels in the heart were determined using the method reported by Ellman[33] modified by Jollow *et al.*[34] The method was based on the development of a yellow colour when DTNB (5,5′-dithiobis-2-nitrobenzoic acid) was added to compounds containing sulfhydryl groups. Five hundred microliters of tissue homogenates in phosphate buffer were added to 3 ml of 4% sulfosalicylic acid. The mixture was centrifuged at 3500*g* for 10 min. Five hundred microliters of supernatants were taken and added to Ellman's reagent. The absorbance was measured at 412 nm after 10 min. The total GSH content in the heart was expressed as μg per mg of protein.

– The heart non-protein thiol (NPSH) levels were determined using the method reported by Ellman.[33] A 500 μl aliquot of the supernatant was mixed with 10% trichloroacetic acid (1 v/1 v). After centrifugation, the protein pellet was discarded and free-SH groups were determined in a clear supernatant. A 100 μl aliquot of the supernatant was added to 850 μl of 1 M potassium phosphate buffer and to 50 μl of DTNB (10 mM). The absorbance of the colorimetric reaction was

measured at 412 nm. The total NPSH content was expressed as nmol per mg of protein.

– The ascorbic acid level was performed as described by Jacques-Silva *et al.*[35] Proteins in the heart were precipitated in 10 volumes of a cold 4% trichloroacetic acid solution. An aliquot of supernatant (300 μl) was adjusted to a final volume of 1 ml and incubated at 85 °C for 30 min, and then 1 ml of H_2SO_4 65% (v/v) was added to the medium. The reaction product was determined using a color reagent containing 4.5 mg ml^{-1} dinitrophenyl hydrazine and copper sulfate (0.075 mg ml^{-1}). The data were expressed as μmol of ascorbic acid per mg of protein.

Heart enzymatic antioxidant activities. – Catalase (CAT) activity was determined by the method of Aebi[36] using hydrogen peroxide (H_2O_2) as a substrate. A decrease in absorbance due to H_2O_2 degradation was monitored spectrophotometrically at 240 nm for 1 min and the enzyme activity was expressed as μmoles of H_2O_2 consumed per minute per milligram of protein.

– Glutathione peroxidase (GPx) activity was measured according to Flohe and Gunzler.[37] The enzyme activity was expressed as nmoles of GSH oxidized per min per mg protein.

– Superoxide dismutase (SOD) activity was estimated according to Beauchamp and Fridovich.[38] The reaction mixture contained heart homogenates in potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM L-methionine, 2 μM riboflavin and 75 mM Nitro Blue Tetrazolium (NBT). The developed blue color of the reaction was measured at 560 nm. Units of SOD activity were expressed as the amount of enzymes required to inhibit the reduction of NBT by 50% and the activity was expressed as units per mg of protein.

Biomarkers of cardiotoxicity. Plasma lactate dehydrogenase (LDH) and creatine kinase isoenzyme (CK-MB) activities were determined according to standard methods by using diagnostic kits from Biomaghreb (Tunisia, Ref 20011 and 20063 respectively).

Lipid profile in plasma. Triglyceride, total cholesterol, HDL-Cholesterol levels were determined by using kits from Biomaghreb (Tunisia, Ref 20131, 20111, 20113,) respectively. The low density lipoprotein cholesterol (LDL-C) fraction was determined according to the Friedewald equation:[39]

$$[\text{LDL-Cholesterol}] = \text{Total cholesterol} - [(\text{Triglyceride}/5) + \text{HDL-Cholesterol}]$$

TC/HDL-C and LDL-C/HDL-C ratios were also calculated. They are considered by Reaven[40] as the pertinent indices of the incidence of cardiovascular risk.

DNA fragmentation assay in the heart

The DNA was extracted according to the standard procedures using commercially available diagnostic kits (Pure Link Genomic DNA Invitrogen ref K 182001). To verify the extent of DNA damage in the heart, we performed the DNA smear technique. It consisted of electrophoresis of full genomic extracted DNA resolved in agarose gel. The gel was observed under an ultraviolet lamp and photographed.

Histopathological studies

Some portions of the heart were placed in 10% of buffered formalin solution for 48 hours. The specimens were washed and dehydrated through a graded series of ethanol. Then they were embedded in paraffin. Blocks were made and sectioned at a thickness of 5 μm and stained with hematoxylin and eosin, then examined under light microscopy and fitted with a Canon Power Shot camera (A640) to capture images for histological studies.

Statistical analysis

The data were analyzed using the statistical package program Stat view 5 Software for Windows (SAS Institute, Berkley, CA). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) test as a *post hoc* test for comparison between groups. The student unpaired *t*-test was also used when comparison between two groups was required. All values were expressed as means ± S.E. The 0.05 level was selected as the point of minimal statistical significance.

Results

Analytical parameters of EVOO and its fractions

EVOO, OOLF and OOHF analytical parameters (fatty acid and antioxidant composition) are presented in Table 2. Extra virgin olive oil and its lipophilic fraction contained 15.98 and 15.93% of saturate (palmitic and stearic acids), 67.23 and 66.74% of monounsaturate (mainly oleic acid), 17.27 and 16.73% of polyunsaturate fatty acids. Some significant differences were noted in the amount of total phenols in our tested oil. In fact, EVOO

Table 2 Mean values of analytical parameters, fatty acid composition and antioxidant content of EVOO, OOHF and OOLF fractions

Analytical parameters	EVOO	OOHF	OOLF
Fatty acid (%)			
Palmitic acid (C16:0)	12.68 ± 0.12	—	12.41 ± 0.15
Palmitoleic acid (C16:1w7)	0.54 ± 0.04	—	0.70 ± 0.03
Stearic acid (C18:0)	2.80 ± 0.06	—	2.97 ± 0.01
Oleic acid (C18:1w9)	66.35 ± 0.39	—	65.69 ± 0.04
Linoleic acid (C18:2w6)	16.03 ± 0.41	—	16.49 ± 0.03
Linolenic acid (C18:3w3)	0.70 ± 0.03	—	0.77 ± 0.05
Arachidonic acid (C20:0)	0.44 ± 0.02	—	0.48 ± 0.01
Gadoleic acid (C20:1w-9)	0.34 ± 0.01	—	0.35 ± 0.01
SFA	15.98 ± 0.13	—	15.93 ± 0.14
MUFA	67.23 ± 0.37	—	66.74 ± 0.23
PUFA	16.73 ± 0.43	—	17.27 ± 0.08
MUFA/PUFA	4.02 ± 0.13	—	3.86 ± 0.03
Antioxidant content (mg kg^{-1})			
Chlorophylls	7.40 ± 0.06	—	7.30 ± 0.02
β-carotene	9.17 ± 0.51	0.41 ± 0.11	10.66 ± 0.89
Total polyphenols	486.01 ± 41.68	145.59 ± 2.48	396.92 ± 18.53
α-tocopherol	310.66 ± 18.81	ND	299.16 ± 13.98
β-tocopherol	46.26 ± 10.01	ND	—

PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; SFA, saturated fatty acids. ND: not determined. —: absent.

and OOLF contained high amounts of phenols (486 and 396.92 mg kg^{-1}) respectively, while OOHF contained less quantities (145.59 mg kg^{-1}). EVOO and OOLF presented the same amount of α-tocopherol while OOHF was deprived from this component (Table 2).

Evaluation of body and absolute heart weights

Death was not observed during the experimental period. As shown in Table 3, the present data recorded a significant decrease in the body weight gain and in the absolute heart weight of rats treated with both $AlCl_3$ and ACR when compared to controls. When EVOO, OOLF and OOHF were administered to treated rats, a recovery occurred in the body and the absolute heart weight (Table 3). These findings indicated that free radicals released in the heart were effectively scavenged by EVOO and its two fractions.

Estimation of MDA, AOPP and non-enzymatic antioxidant levels in the heart

In the $AlCl_3$ and ACR-treated rats, a significant increase in lipid peroxidation (90%) and protein oxidation (78%) was observed when compared to those of controls (Table 4). Furthermore, a significant decrease in the MDA and AOPP levels was observed after co-administration of EVOO, OOLF or OOHF to $AlCl_3$ and ACR treated rats.

A significant decline in the GSH (20%), NPSH (47%) and vitamin C (28%) levels was found in the rats co-exposed to $AlCl_3$ and ACR (Table 4). Our findings indicated that free radicals released in the heart were effectively scavenged after supplementation of EVOO or OOLF.

Heart enzymatic antioxidant activities

The activities of the cardiac antioxidant enzymes are presented in Fig. 1. Co-exposure of rats to both aluminum and acrylamide produced a significant decrease in the heart catalase, glutathione peroxidase and superoxide dismutase enzyme activities when compared with those of the control group ($p < 0.001$). EVOO and its two fractions restored the activities of these enzymes ($p < 0.01$) to near control values (Fig. 1).

Plasma biomarkers of cardiotoxicity

Fig. 2 shows plasma LDH and CK activities of control and experimental rats. A significant increase in the activities of these enzymes by 83 and 120% was observed in the co-exposed

Table 3 Initial and final body weights, absolute heart weight, daily food and water consumption in controls and rats treated with $AlCl_3$ and ACR during 21 days. Corrective effects of EVOO and its fractions (OOLF and OOHF)

	Initial body weight (g)	Final body weight (g)	Absolute heart weight (g)	Food consumption (g per day per rat)	Drinking water intake (ml per day per rat)
Controls	161 ± 2.88	180.33 ± 6.81	0.621 ± 0.027	12.39 ± 2.23	27.92 ± 4.32
$AlCl_3$ + ACR	161.16 ± 3.97	156.52 ± 4.08***	0.573 ± 0.029***	10.28 ± 2.27**	20.84 ± 5.28***
$AlCl_3$ + ACR + EVOO	162 ± 3.81	183.4 ± 4.51$^{+++\S}$	0.643 ± 0.014^{+++}	12.92 ± 2.58^{++}	24.34 ± 2.16*$^{+}$
$AlCl_3$ + ACR + OOLF	163.52 ± 2.74	179.52 ± 4.53$^{+++¥}$	0.633 ± 0.011^{+++}	12.63 ± 2.33^{++}	26.87 ± 3.66^{++}
$AlCl_3$ + ACR + OOHF	163.76 ± 3.49	178.49 ± 5.12^{+++}	0.624 ± 0.013^{+++}	11.87 ± 1.96^{+}	25.36 ± 2.55*$^{++}$
EVOO	161.5 ± 3.81	185.51 ± 5.32	0.666 ± 0.009	11.75 ± 0.89	26.17 ± 2.03
OOLF	163.25 ± 3.87	182.75 ± 4.62	0.629 ± 0.013	11.66 ± 1.72	27.75 ± 2.59
OOHF	164.22 ± 2.98	180.96 ± 4.46	0.635 ± 0.011	11.53 ± 1.35	25.66 ± 3.21

Values are means ± SE for six rats in each group. ($AlCl_3$ + ACR); ($AlCl_3$ + ACR + EVOO); ($AlCl_3$ + ACR + OOLF); ($AlCl_3$ + ACR + OOHF); EVOO; OOLF and OOHF treated groups *vs.* control group: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. ($AlCl_3$ + ACR + EVOO); ($AlCl_3$ + ACR + OOLF); ($AlCl_3$ + ACR + OOHF) groups *vs.* ($AlCl_3$ + ACR) group: $^{+}p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.001$. ($AlCl_3$ + ACR + EVOO); ($AlCl_3$ + ACR + OOLF) groups *vs.* ($AlCl_3$ + ACR + OOHF) group: $^{\S}p < 0.05$. ($AlCl_3$ + ACR + OOLF) group *vs.* ($AlCl_3$ + ACR + EVOO) group: $^{¥}p < 0.05$.

Table 4 MDA, AOPP, GSH, NPSH and vitamin C levels in the heart of controls and rats treated with $AlCl_3$ and ACR. Corrective effects of EVOO and its fractions (OOLF and OOHF)

	MDA[a]	AOPP[b]	GSH[c]	NPSH[b]	Vitamin C[d]
Controls	1.37 ± 0.21	0.14 ± 0.01	5.59 ± 0.12	22.22 ± 1.33	1.52 ± 0.04
$AlCl_3$ + ACR	2.61 ± 0.11***	0.25 ± 0.02***	4.51 ± 0.31***	11.69 ± 1.31***	1.09 ± 0.12***
$AlCl_3$ + ACR + EVOO	1.32 ± 0.11$^{+++\S\S\S}$	0.15 ± 0.01$^{+++\S}$	5.68 ± 0.16$^{+++\S}$	20.69 ± 0.61$^{+++\S}$	1.58 ± 0.13^{+++}
$AlCl_3$ + ACR + OOLF	1.13 ± 0.06$^{+++\S\S\S¥¥}$	0.13 ± 0.01$^{+++\S¥¥}$	5.62 ± 0.26^{+++}	20.91 ± 0.99$^{+++\S}$	1.51 ± 0.02^{+++}
$AlCl_3$ + ACR + OOHF	1.65 ± 0.09^{+++}	0.16 ± 0.01^{+++}	5.21 ± 0.25^{++}	18.99 ± 0.93^{+++}	1.46 ± 0.06^{++}
EVOO	1.26 ± 0.16	0.14 ± 0.01	5.52 ± 0.14	21.37 ± 0.59	1.50 ± 0.05
OOLF	1.06 ± 0.07	0.15 ± 0.01	5.57 ± 0.26	21.45 ± 1.31	1.53 ± 0.06
OOHF	1.32 ± 0.08	0.14 ± 0.01	5.51 ± 0.09	21.28 ± 0.62	1.52 ± 0.11

[a] nmoles of MDA per mg of protein. [b] nmoles per mg of protein. [c] μg mg^{-1} protein. [d] μmol mg^{-1} protein. Values are means ± SE for six rats in each group. ($AlCl_3$ + ACR); ($AlCl_3$ + ACR + EVOO); ($AlCl_3$ + ACR + OOLF); ($AlCl_3$ + ACR + OOHF); EVOO; OOLF and OOHF treated groups *vs.* control group: ***$p < 0.001$. ($AlCl_3$ + ACR + EVOO); ($AlCl_3$ + ACR + OOLF); ($AlCl_3$ + ACR + OOHF) groups *vs.* ($AlCl_3$ + ACR) group: $^{++}p < 0.01$; $^{+++}p < 0.001$. ($AlCl_3$ + ACR + EVOO); ($AlCl_3$ + ACR + OOLF) groups *vs.* ($AlCl_3$ + ACR + OOHF) group: $^{\S}p < 0.05$; $^{\S\S\S}p < 0.001$. ($AlCl_3$ + ACR + OOLF) group *vs.* ($AlCl_3$ + ACR + EVOO) group: $^{¥¥}p < 0.01$.

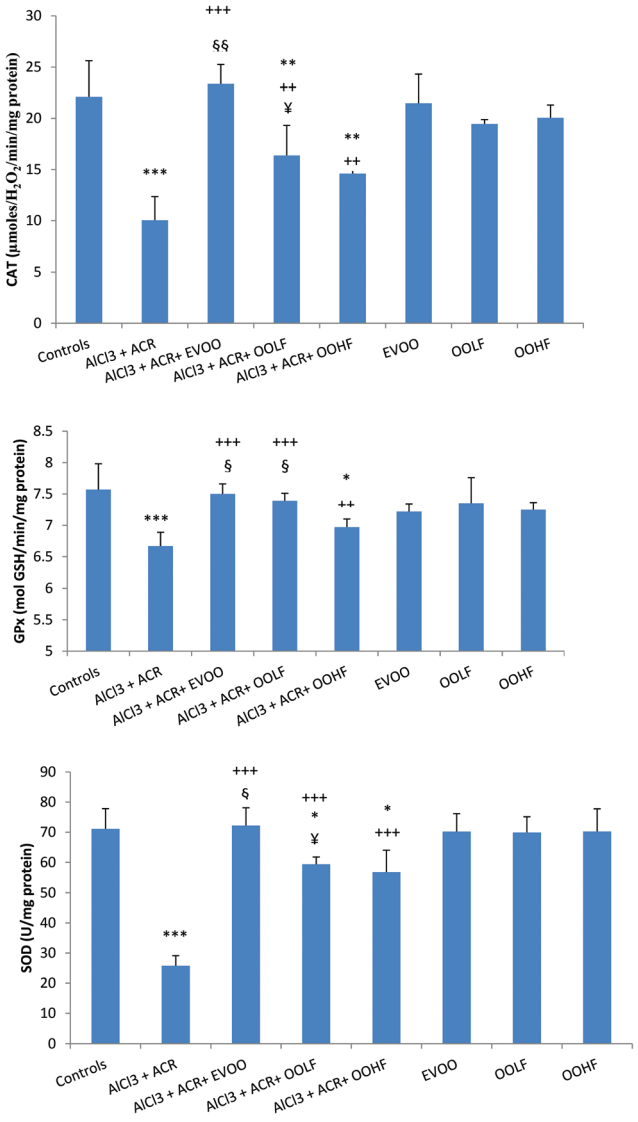

Fig. 1 Anti-oxidant enzyme activities of controls and rats treated with $AlCl_3$ and ACR. Corrective effects of EVOO and its fractions (OOLF and OOHF). Values are expressed as means ± S.D. for six rats in each group. ($AlCl_3$ + ACR); ($AlCl_3$ + ACR + EVOO); ($AlCl_3$ + ACR + OOLF); ($AlCl_3$ + ACR + OOHF); EVOO; OOLF and OOHF treated groups *vs.* control group: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. ($AlCl_3$ + ACR + EVOO); ($AlCl_3$ + ACR + OOLF); ($AlCl_3$ + ACR + OOHF) groups *vs.* ($AlCl_3$ + ACR) group: $^{++}p < 0.01$; $^{+++}p < 0.001$. ($AlCl_3$ + ACR + EVOO); ($AlCl_3$ + ACR + OOLF) groups *vs.* ($AlCl_3$ + ACR + OOHF) group: $^{\S}p < 0.05$; $^{\S\S}p < 0.01$. ($AlCl_3$ + ACR + OOLF) group *vs.* ($AlCl_3$ + ACR + EVOO) group: $^{¥}p < 0.05$.

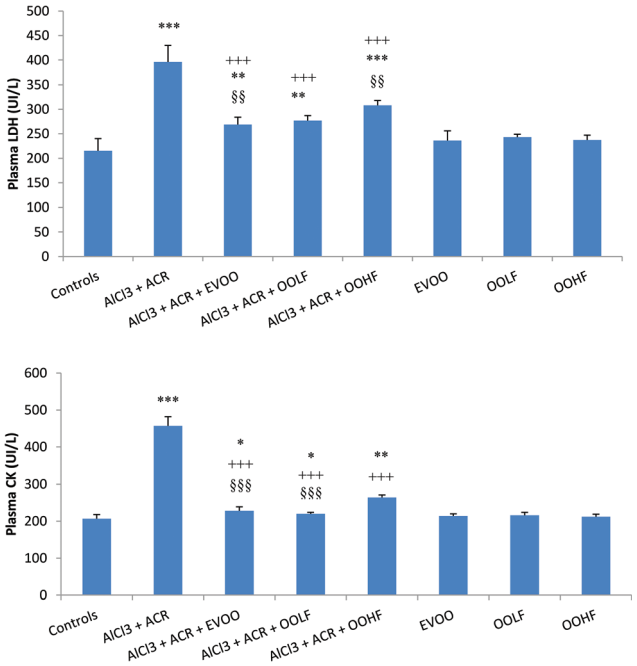

Fig. 2 Cardiac markers (LDH and CK) in control and treated rats. Protective effects of EVOO and its fractions (OOLF and OOHF). Values are expressed as means ± S.D. for six rats in each group. ($AlCl_3$ + ACR); ($AlCl_3$ + ACR + EVOO); ($AlCl_3$ + ACR + OOLF); ($AlCl_3$ + ACR + OOHF); EVOO; OOLF and OOHF treated groups *vs.* control group: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. ($AlCl_3$ + ACR + EVOO); ($AlCl_3$ + ACR + OOLF); ($AlCl_3$ + ACR + OOHF) groups *vs.* ($AlCl_3$ + ACR) group: $^{+++}p < 0.001$. ($AlCl_3$ + ACR + EVOO); ($AlCl_3$ + ACR + OOLF) groups *vs.* ($AlCl_3$ + ACR + OOHF) group: $^{\S\S}p < 0.01$; $^{\S\S\S}p < 0.001$.

group when compared to controls. Extra virgin olive oil and its lipophilic fraction restored these parameters to normal values.

Lipid profile

Table 5 shows the levels of plasma lipid profile in the control and experimental rats. In the $AlCl_3$ and ACR-treated rats, TC and LDL-C levels were increased by 31% and 33%, respectively, whereas the HDL-C and TG levels decreased by 55 and 41%, respectively.

As a consequence, the incidences of cardiovascular disease risk index were significantly lowered mainly by oral administration of EVOO and OOLF (Table 5).

Effect of $AlCl_3$ and ACR on heart DNA damage

As shown in Fig. 3, a smear (hallmark of necrosis) without ladder formation on agarose gel, indicating random DNA degradation, was observed through the DNA heart lane of rats treated with both $AlCl_3$ and ACR. Co-treatment with EVOO and its two fractions (OOLF, OOHF) exerted a protective effect against treatment by reducing the smear formation in the heart.

Effect of $AlCl_3$ and ACR on histopathological studies

Fig. 4A shows the appearance of normal cardiac muscle fibers. In $AlCl_3$ and ACR treated rats, there were a marked vascular congestion and inflammatory cell infiltration (Fig. 4B). EVOO and its fractions (OOLF, OOHF) restored the aspect of cardiac muscle which appeared similar to that of controls (Fig. 4C–E) respectively.

Table 5 Lipid profile in plasma of controls and rats treated with $AlCl_3$ and ACR. Corrective effects of EVOO and its fractions (OOLF and OOHF)

Parameters & treatment	Triglycerides[a]	Total-C[a]	HDL-C[a]	LDL-C[a]	T-C/HDL-C[a]	LDL-C/HDL-C[a]
Controls	85.08 ± 3.29	62 ± 3.08	19.03 ± 2.13	26.38 ± 2.34	3.26 ± 0.26	1.40 ± 0.18
$AlCl_3$ + ACR	49.88 ± 8.26***	81.33 ± 4.85***	8.45 ± 1.65***	35.22 ± 1.76***	9.23 ± 0.76***	3.93 ± 0.49***
$AlCl_3$ + ACR + EVOO	87.43 ± 4.76^{+++}§§§	48.56 ± 4.83^{+++}§§§	15.96 ± 2.68^{+++}	20.70 ± 1.02^{+++}§§	3.07 ± 0.27^{+++}§§	1.32 ± 0.24^{+++}§
$AlCl_3$ + ACR + OOLF	72.21 ± 3.27^{+++}§§¥¥¥	59.21 ± 1.52^{+++}§§§¥¥	16.64 ± 2.79^{+++}	23.41 ± 1.49^{+++}¥	3.62 ± 0.57^{+++}§	1.43 ± 0.26^{+++}
$AlCl_3$ + ACR + OOHF	63.08 ± 2.09^{++}	65.79 ± 1.82^{+++}	14.89 ± 1.29^{+++}	24.86 ± 1.98^{+++}	4.44 ± 0.46^{+++}	1.69 ± 0.29^{+++}
EVOO	86.13 ± 8.05	63.37 ± 4.37	18.76 ± 2.24	27.76 ± 3.01	3.66 ± 0.66	1.61 ± 0.37
OOLF	82.65 ± 4.79	64.14 ± 1.98	18.57 ± 2.21	26.89 ± 1.03	3.48 ± 0.32	1.46 ± 0.17
OOHF	81.12 ± 3.747	62.61 ± 2.48	18.28 ± 1.28	25.73 ± 1.72	3.43 ± 0.11	1.41 ± 0.09

HDL-C: high density lipoprotein-cholesterol; LDL-C: low density lipoprotein-cholesterol. [a] mg dl^{-1}. Values are means ± SE for six rats in each group. ($AlCl_3$ + ACR); ($AlCl_3$ + ACR + EVOO); ($AlCl_3$ + ACR + OOLF); ($AlCl_3$ + ACR + OOHF); EVOO; OOLF and OOHF treated groups *vs.* control group: ***$p < 0.001$. ($AlCl_3$ + ACR + EVOO); ($AlCl_3$ + ACR + OOLF); ($AlCl_3$ + ACR + OOHF) groups *vs.* ($AlCl_3$ + ACR) group: $^{+}p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.001$. ($AlCl_3$ + ACR + EVOO); ($AlCl_3$ + ACR + OOLF) groups *vs.* ($AlCl_3$ + ACR + OOHF) group: $^{§}p < 0.05$; $^{§§}p < 0.01$; $^{§§§}p < 0.001$. ($AlCl_3$ + ACR + OOLF) group *vs.* ($AlCl_3$ + ACR + EVOO) group: $^{¥}p < 0.05$. $^{¥¥}p < 0.01$; $^{¥¥¥}p < 0.001$. Lipid profile of controls, $AlCl_3$ + ACR and $AlCl_3$ + ACR + EVOO was reported in our previous paper.[59]

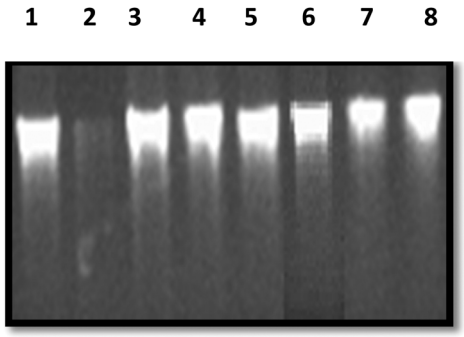

Fig. 3 Electrophoresis of DNA isolated from livers of control and experimental rats was loaded into 1% agarose gel. Lane 1: DNA isolated from normal heart; lane 2: DNA isolated from $AlCl_3$ and ACR heart samples; lane 3: DNA isolated from $AlCl_3$ and ACR heart samples supplemented with EVOO; lane 4: DNA isolated from $AlCl_3$ and ACR supplemented with OOLF heart samples; lane 5: DNA isolated from $AlCl_3$ and ACR supplemented with OOHF heart sample; lane 6: DNA isolated from EVOO heart samples; lane 7: DNA isolated from OOLF heart samples; lane 8: DNA isolated from OOHF heart samples.

Discussion

Accumulation of acrylamide and aluminum in food is a major source of human exposure and hence a threat to human health. Although numerous reports were available on the individual effects of $AlCl_3$ and ACR, their combined exposure which might cause more severe toxic effects in the heart had not yet been studied. Cardiovascular diseases like hypertension, aneurysms, thrombosis, heart attack, stroke, *etc*. grew with the increase of life expectancy. So, in this study extra virgin olive oil and its hydrophilic and lipophilic fractions were tested for their lipid-lowering, anti-oxidative, and cardioprotective effects against oxidative damage induced by both aluminum and acrylamide in adult rats.

Concurrent exposure to these toxicants might produce additive or synergistic interactions or even new effects that are not seen in a single component exposure. Biomarkers of heart injuries can reflect physiological and morphological changes in cells and tissue resulting from co-exposure. A significant decrease of body and absolute heart weights was observed in co-exposed rats when compared to controls. The reduction could be explained either by a significant decrease of feed and water consumption as reported by us or/and by the toxicity induced by xenobiotics according to Grance *et al.*[41] Our results concerning disturbances in the total body weights were consistent with previous studies of Wang *et al.*[42] and Zhu *et al.*[43] after respective treatments by ACR and $AlCl_3$. Co-administration of extra virgin olive oil and its lipophilic and hydrophilic fractions attenuated cardiotoxicity as shown by morphological changes (Table 3). When EVOO was supplemented to the treated rats, recovery occurred in the body weight and there were no significant changes in BW between controls and rats treated with EVOO. So, the beneficial effects of EVOO intake on the prevention of cardiovascular diseases cannot be attributed only to the content of oleic acid because the hydrophilic fraction did not contain fatty acids.

The heart is composed primarily of long-lived, post-mitotic cells, which prefer fatty acids as substrate for energy production, so it becomes more susceptible to oxidative damage than other tissues.[44] Under oxidative stress, cells display various dysfunctions due to the lesions caused by reactive oxygen species (ROS) in DNA, proteins and lipids.[45] Increased free radical production may be responsible for the observed membrane damage as evidenced by the elevated lipid peroxidation in terms of MDA reactive substances in the co-exposed rats. ROS causing damage to lipids can also cause deleterious effects on proteins. Their oxidation can generate the stable as well as reactive products that can generate additional radicals upon reaction with transition metal ions. Our results showed an increase of AOPP levels in cardiac tissue of rats co-treated with both aluminum and acrylamide. Co-administration of EVOO and OOLF was more effective in decreasing MDA and AOPP levels in the heart by protecting the critical cellular lipids and proteins from oxidation. This could be explained by the fact that the consumption of extra virgin olive oil increases

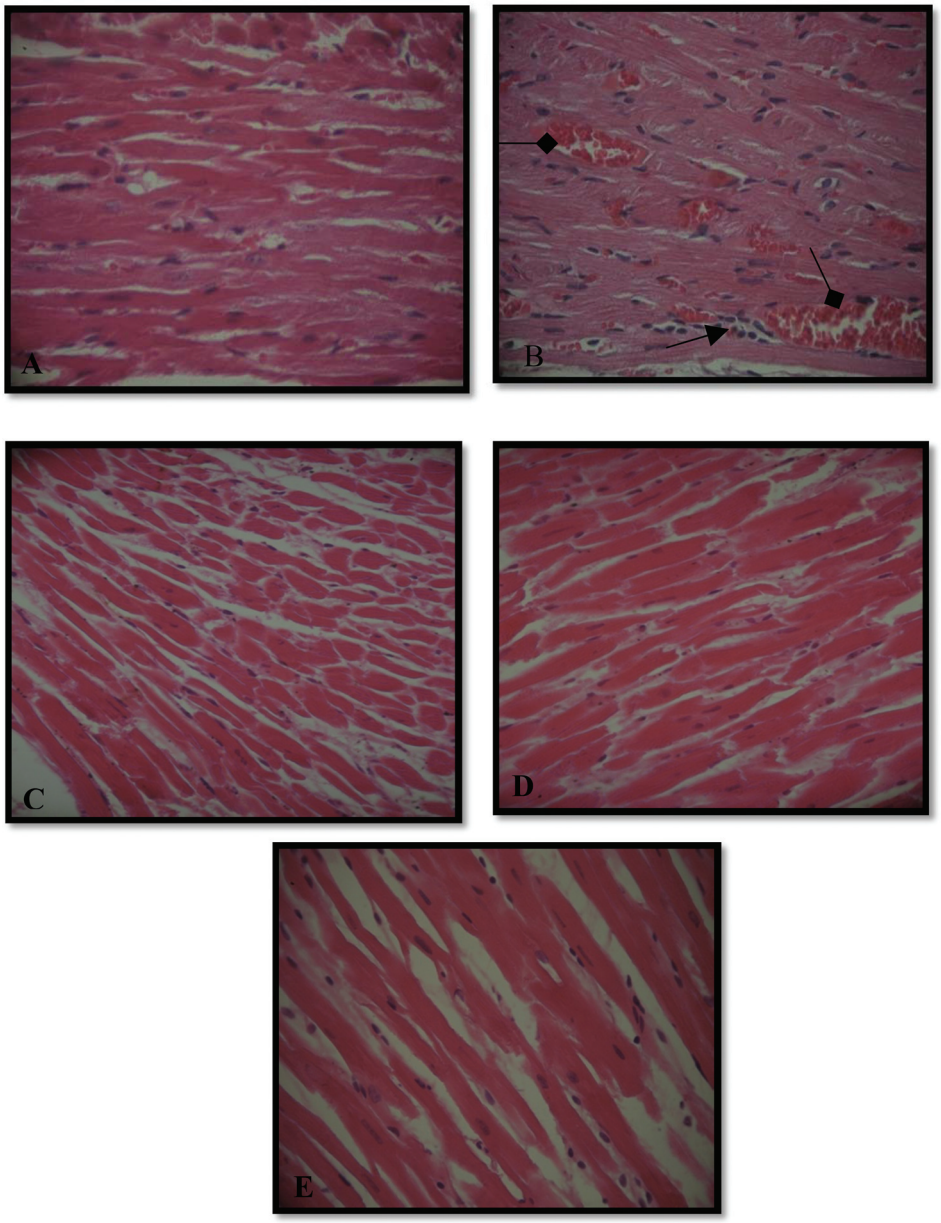

Fig. 4 Heart histological sections of controls (A), $AlCl_3$ and ACR (B), combined group plus EVOO (C); combined group plus OOLF (D); combined group plus OOHF (E). Arrows indicate: ⟶ leucocyte inflammatory cells; ⟶◆ vascular congestion; optic microscopy: H&E ×400.

the levels of oleic acid in cell membranes, which helps to regulate the structure of membrane lipids through control of signal-mediated G protein, causing a reduction in blood pressure.[46] On the other hand, the beneficial effects of EVOO in the Mediterranean diet could be attributed not only to the close relationship between unsaturated and saturated fatty acids, but also to the antioxidant properties of its phenolic compounds. Therefore, the hydrophilic fraction contained only natural anti-oxidants including β-carotenes and polyphenols which revealed a protective role against oxidative stress caused by concomitant exposure to $AlCl_3$ and ACR without reaching normal values.

Cellular redox reactions are involved in metabolic, signaling and transcriptional processes with thiols in the form of cysteine residues as vital players in redox sensing and regulation.[47] GSH acts as a free radical scavenger and protects the heart from oxidative stress.[48] Co-exposure to $AlCl_3$ and ACR was characterized by the depletion of non-enzymatic antioxidants in the heart including glutathione (GSH), non-protein thiols (NPSH) and vitamin C. The reduced levels of GSH and NPSH could be the result of either an increased utilization for conjugation and/or their participation as antioxidants in terminating free radical products. However, oral administration of EVOO and its two fractions to both aluminum and acryl-

amide-treated rats caused a modulation in the levels of non-enzymatic antioxidant and lipid peroxidation, which might result from the stabilization of the plasma membrane as well as the repair of the cardiac tissue damage.

An antioxidant defense system, mainly involved in the scavenging reactive oxygen species (ROS) prevents oxidative stress. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities, an excellent team of defense against ROS,[49] were decreased. This reduction indicates the excessive utilization of these antioxidants in order to scavenge free radicals. Our results were in accordance with previous reports of Azad *et al.*[50] who have shown an increase in myocardial malonyldialdehyde and a decrease in antioxidant enzyme activities after aluminium phosphide treatment. Thus, our study indicated that EVOO and its two fractions provided protection against aluminum and acrylamide-induced oxidative stress and cardiac dysfunction. This improvement was more pronounced in rats treated with EVOO than OOLF and OOHF used separately reflecting the synergic effect of the two fractions in order to restore the activities of anti-oxidant enzymes. Some studies have demonstrated that EVOO administration may provide protective effects against oxidative stress in tissues by increasing the activity of antioxidant enzymes.[51,52]

When myocardial cells are damaged or destroyed due to the deficiency of oxygen supply or glucose, the cell membrane becomes permeable or may be disrupted leading to the leakage of enzymes. Creatinine kinase (CK) and lactate dehydrogenase (LDH) activities in the heart reflect the alterations of membrane integrity and the degree of myocardial injury.[53,54] Several studies have consistently reported the elevated activities of these enzymes in the serum of experimental animals immediately after myocardial infarction.[55] Our results showed that CK and LDH activities in the plasma of treated rats were clearly increased indicating myocardial injury. Thus, EVOO and its two fractions prevented the leakage of these biomarkers from the heart into blood due to their high antioxidant capacity leading to membrane stabilizing action thereby reducing cardiac tissue damage. Indeed, EVOO contains a considerable amount of oleuropein, hydroxytyrosol, tyrosol and caffeic acid which have potent inhibitory effects against ROS.[56,57]

In addition, circulating lipids, lipoproteins and cardiac lipids play a crucial role in the pathogenesis of myocardial disorder. Thus, the change in lipid profile can lead to various diseases such as atherosclerosis, lung fibrosis, inflammatory bowel disease and rheumatoid arthritis.[58] Co-exposure to aluminum and acrylamide caused an important increase in the plasma TC and LDL-C levels and a decrease in HDL-C and TG, which indicated a change in the permeability of hepatic cells as reported in our previous study.[59] The decline of TC and LDL-C levels and the increase of HDL-C and TG levels have been linked to a low risk of cardiovascular disease.[60] We found that EVOO was effective in ameliorating aluminum and acrylamide induced myocardial damage. Even though EVOO and OOLF fatty acid analyses revealed the same amount of MUFA, they showed a higher content of unsaponifiable components like polyphenols in EVOO, which might contribute to olive oil's beneficial effect. This beneficial role of EVOO according to Taamalli *et al.*[61] and Ben Hassine *et al.*[19] might be attributed to apigenin rutinoside, elenolic acid, luteolin, flavonoid, and hydroxytyrosol (HTy) compounds found in Chetoui extra virgin olive oil.

To substantiate the biochemical findings, a histological examination of the heart was undertaken. Our study showed an altered cardiac histoarchitecture in treated rats, such as inflammation and a marked vascular congestion. EVOO and its two fractions protected the myocardium against aluminum and acrylamide induced cardiac damage.

When redox-active species are in excess, they may cause DNA damage, repress the activity of cellular enzymes, and induce genotoxicity and cell death.[62,63] Genomic DNA analysis by agarose gel electrophoresis confirmed that co-exposure to aluminum and acrylamide mediated apoptosis and displayed marked DNA damage. Our results were in agreement with Zamorano-Ponce *et al.*[15] who have shown that acrylamide induces genotoxicity in cells by decreasing the oxidative defense system. It is known that the ladder pattern of fragmentation is generally observed in apoptosis due to endonuclease mediated internucleosomal fragmentation of DNA. Co-administration of EVOO or its two fractions (OOLF and OOHF) significantly reduced $AlCl_3$ and ACR induced DNA damage *via* their strong antioxidant activity and thereby restored the DNA integrity of myocardial cells. Besides the beneficial effect of their fatty acids, extra virgin olive oil and its two fractions provided a rich variety of natural antioxidants including carotenoids and polyphenols.

Conflict of interest

The authors declare that they have no competing interests to disclose.

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