Extra Virgin Olive Oil Reduced Polyunsaturated Fatty Acid and Cholesterol Oxidation in Rodent Liver: Is This Accounted for Hydroxytyrosol-Fatty Acid Conjugation?
Yiu Lee, Celine Crauste, Hualin Wang, Ho Hang Leung, Joseph Vercauteren, Jean-Marie Galano, Camille Oger, Thierry Durand, Jennifer Man-Fan Wan, Jetty Chung-Yung Lee

To cite this version:
Yiu Lee, Celine Crauste, Hualin Wang, Ho Hang Leung, Joseph Vercauteren, et al.. Extra Virgin Olive Oil Reduced Polyunsaturated Fatty Acid and Cholesterol Oxidation in Rodent Liver: Is This Accounted for Hydroxytyrosol-Fatty Acid Conjugation?. Chemical Research in Toxicology, American Chemical Society, 2016, 29 (10), pp.1689-1698. 10.1021/acs.chemrestox.6b00214. hal-01622331

HAL Id: hal-01622331
https://hal.umontpellier.fr/hal-01622331
Submitted on 29 Mar 2018

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
Extra Virgin Olive Oil Reduced Polyunsaturated Fatty Acid and Cholesterol Oxidation in Rodent Liver: Is This Accounted for Hydroxytyrosol-Fatty Acid Conjugation?

Yiu Yiu Lee,† Céline Crauste,‡ Hualin Wang,† Ho Hang Leung,† Joseph Vercauteren,‡ Jean-Marie Galano,‡ Camille Oger,‡ Thierry Durand,‡ Jennifer Man-Fan Wan,† and Jetty Chung-Yung Lee*†

†School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong SAR
‡Institut des Biomolécules Max Mousseron, UMR 5247, CNRS, Université de Montpellier & ENSCM (School of Chemistry), Faculté de Pharmacie, 15 Av. Charles Flahault, 34093 Montpellier cedex 05, France

Supporting Information

ABSTRACT: The effects of extra virgin olive oil (EVOO) and carbon tetrachloride (CCl₄) induced oxidative stress in rats were determined by the generation of isoprostanoids. These are known to be robust biomarkers to evaluate nonenzymatic and free radical related oxidation. Other oxidative stress biomarkers such as hydroxyeicosatetraenoic acid products (HETEs) and cholesterol oxidation products (COPs) were also determined. The rodents received a control diet, high-fat diet (20% w/w) composed of extra virgin olive oil (EVOO), corn oil (CO), or lard, and high-fat diets with CCl₄ insult throughout the experimental period. The EVOO diet was found to suppress the formation of isoprostanoids and COPs compared to that of the control. EVOO also had a high total phenolic content and antioxidant activity compared to those of CO and lard and may be contributed to by the hydroxytyrosol component conjugated to fatty acids (HT-FA). This is the first study to identify HT-FA in EVOO, and it was 4-fold higher than that of olive oil, whereas none was found in corn oil. Furthermore, the EVOO diet showed reduced liver lipid vesicles in CCl₄ treated rats compared to that of the control. However, liver toxicity measurements of AST (aspartate transaminase) and ALT (alanine transaminase) activities showed augmentation with CCl₄ treatment but were not alleviated by the diets given. Our findings suggest that EVOO is a daily functional food capable of enhancing the antioxidant system for liver protection; the effect is potentially attributed to the phenolic and lipophenolic (phenol conjugated by fatty acids) content.

1. INTRODUCTION

Dietary extra virgin olive oil (EVOO) has shown protection against coronary heart disease (CHD) by lowering total cholesterol and low-density lipoprotein (LDL). Also, it reduced blood pressure and insulin sensitivity in healthy and type 2 diabetic patients. Epidemiological studies have also shown that individuals who had low saturated fats and high intake of olive oil displayed lower rates of cardiovascular disease (CVD) and cancer. In addition, an EVOO rich Mediterranean diet was more effective in primary and secondary prevention of CVD than the diet designed by the United States cholesterol education program. In animal studies, 5–10% of EVOO enriched diets with low cholesterol level halted the progression of induced atherosclerosis in rabbits and in female apolipoprotein E knockout (ApoE⁻/⁻) mice. The fatty acid composition of EVOO is mainly oleic acid, an omega-9 monounsaturated fatty acid (MUFA), along with polar components such as polyphenols that are widely known to be involved in antioxidant activity. To date, it is unknown how the polyphenol is delivered in vivo for its activity. It has been shown that polyphenols in the gut increase catabolism, hydrolyze bile salt, and modify the enterohepatic circulation. Further, it is suggested that some polyphenols and omega-3 polyunsaturated fatty acid (PUFA) together down-regulate prostaglandin production and regulate inflammation and antioxidant pathways.

Chronic liver diseases, including chronic hepatitis virus B (HBV) and/or hepatitis virus C (HCV) infection, alcoholic liver disease, NAFLD, and primary biliary cirrhosis are major threats to human health in Asia. Furthermore, chronic liver diseases will lead to liver inflammation, hepatocellular dysfunction, activation of hepatic stellate cells, and accumulation of extracellular matrix, also known as liver fibrosis and advanced cirrhosis, and even hepatocellular carcinoma.

It is elucidated that hydroxytyrosol (HT) is the active polyphenol in EVOO, but the underlying mechanisms on its effect and health are still unclear. Recently, HT was found to...
exert antiadipogenic effects in preadipocyte cells. Also, studies have focused on the effect of EVOO in CHD, but those related to the liver, e.g., nonalcoholic fatty liver disease (NAFLD), are scarce. Reports suggest that liver damage arises in part from free radicals of exogenous sources such as alcohol, diet, drugs, and chemical contaminants, therefore, the role of HT in EVOO may have a substantial impact.

It is known that oxidation of omega-6 PUFAs such as arachidonic acid (AA) via a nonenzymatic pathway can generate F2-isoprostanes (with its best known isomer being 15-F2t-isoprostane or 15-F2t-isoP), while incorporation of an enzymatic pathway can release hydroxyeicosatetraenoic acid products (HETEs). Augmentations of these metabolites are toxic and highly associated with inflammation, CHD, and neurodegeneration.20 In addition, resolvins D1 from enzymatic oxidation of docosahexaenoic acid (DHA) showed reduced inflammation in various biological models.23 Moreover, nonenzymatic oxidation of omega-3 PUFAs such as eicosapentaenoic (EPA) and DHA releases F1t-isoprostanes (8-F1t-isoprostane) and other F2t-neuroprostane (F2t-NeuroPs), for example, 4(RS)-4-F2t-Neuroprostanes (4(RS)-4-F2t-NeuroPeptide), respectively. The generation of the latter is desirable as it was recently shown to be cardioprotective.24,25 Nonetheless, earlier studies indicate F4t-NeuroPs to be a robust oxidative stress biomarker for neurodegeneration.26

Oxidation of cholesterol could also take place in the liver to release 7β-hydroxycholesterol (7β-OH and 7-ketocholesterol (7-ketochol) via free radical reaction, and 27-hydroxycholesterol (27-OH) via the cytochrome P450 pathway, and released into blood circulation by LDL. Elevation of these cholesterol oxidation products (COPs) can lead to lysosomal dysfunction and mitochondrial damage in human monocytes and is associated with vascular related disorder such as atherosclerosis.27

In this article, we administered CCl4 to induce hepatotoxicity in a rat model to study the mechanisms of lipid peroxidation and the protective effects of EVOO (predominantly omega-9 MUFA), corn oil (mostly omega-6 PUFA), and lard (higher content of saturated fats) in rodents. It is well established that CCl4 can be metabolized to a trichloromethyl radical via cytochrome P450 isoenzymes and mediate free radical reaction, and lipid peroxidation. Alteration on PUFA and cholesterol by generating unwanted oxidized metabolites leads to liver cell necrosis.28,29 However, some oxidized metabolites are also recognized as lipid mediators that could exert important biological functions.26,30 In addition, we identified that hydroxytyrosol is present in the form of lipophenol derivatives that are covalently conjugated to certain fatty acids in EVOO and olive oil but not corn oil.

2. MATERIALS AND METHODS

2.1. Treatments and Sampling of the Rodents. Five month old male Fischer 344 rats (each weighing approximately 450 g) were obtained from the Animal Resources Centre, Western Australia and bred in the Animal House, School of Biological Sciences, at The University of Hong Kong (HKU) under the approval of Department of Health, Hong Kong and Committee on the Use of Live Animals in Teaching and Research, HKU. All rats were kept individually in autoclaved stainless steel cages in the animal room. The rats experienced a 12 h light−dark cycle with controlled temperatures (22 °C) and humidity (50%). Animals received water ad libitum. After 1 week of acclimatization, the rats were randomly divided into either control (normal chow) or high-fat groups. The diet for high-fat groups was extra virgin olive oil (EVOO), corn oil (CO), and lard. The rats in the high-fat groups were fed with 20% (w/w) of respective oils mixed with normal rodent chow (5053, LabDiet, USA) ad libitum. Animals in the high-fat group were subdivided for CCl4 (+ CCl4) or control (− CCl4) treatments. After 1 week of conditioning feeding, the animals in the CCl4 treatment group were subcutaneously administered with CCl4 (0.1 mL/100 g body weight, i:1 mixed with soybean oil) twice a week (Monday and Thursday) for 4 weeks, at the same time of the day, in order to induce liver injury. The high-fat diets were maintained throughout the experimental period. Twenty-four hours after the last injection, all rats were sacrificed. Blood samples were collected via cardiac puncture and were allowed to clot. The serum was collected by centrifugation (1500g, 5 min, 4 °C) for hepatic toxicity analysis. The liver tissue was excised, washed with butylated hydroxylxylene (BHT) and indomethacin in PBS (pH 7.4), snap frozen in liquid nitrogen, and stored at −80 °C for future assessment of lipid and lipid mediators. The right lobe of the control and dietary oil fed groups were collected and fixed in 10% buffered formalin solution (Surgipath, Germany) for histological staining.

2.2. Measurement of AST and ALT. Liver toxicity tests were performed by measuring the levels of alanine aminotransferase (ALT) and aspartate transaminase (AST) in the serum. ALT substrate solution (2 mM α-ketoglutarate and 200 mM L-alanine in 0.1 M phosphate buffer, pH 7.4) and AST substrate solution (2 mM α-ketoglutarate and 200 mM L- aspartate in 0.1 M phosphate buffer, pH 7.4) were freshly prepared and incubated at 37 °C. A hundred microliters of serum sample were mixed with 0.5 mL of preincubated ALT or AST substrate solution. The mixture was then incubated in a water bath at 37 °C for 30 min. Then, 0.5 mL of 1M 2,4-dinitrophenylhydrazine (DNPH) color reagent was added to the mixture and allowed to stand at room temperature for 30 min. Finally, 5 mL of 0.4 M NaOH was added and incubated for color development. After 5 min of incubation, the mixture absorbance was measured spectrophotometrically using a UV−vis spectrophotometer (Shimadzu, Japan) at 505 nm.

2.3. Liver Histology. The fixed liver tissues were embedded in paraffin and sectioned at 5-μm thickness. Sections were stained with Masson’s trichrome for collagen (Sigma-Aldrich, USA). The stained slides were observed photomicrographically under a Nikon N800 microscope (Nikon, Japan) at 100X magnification.

2.4. Total Tissue Lipid Extraction. Liver tissues stored in −80 °C were weighed and homogenized in Folch solution (chloroform:methanol, 2:1, v/v) spiked with 0.01% BHT, using a polystyrene benchtop homogenizer (T25, ULTRA-TURRAX, IKA) at 24,000 rpm in 2 x 20 s bursts. Sample tubes were placed on ice during homogenization, and the blade was rinsed with Milli-Q water and methanol between samples to prevent cross-contamination. Subsequently, 2 mL of aqueous 0.9% NaCl was added and centrifuged at 3000g for 10 min at 4 °C, to introduce phase separation. The lower phase was collected and dried under nitrogen flux on a 37 °C heating block. Dried lipids were dissolved in 1 mL of methanol containing 0.005% BHT and hydrolyzed by adding 1 mL of 1 M aqueous KOH. Samples were vortexed, purged with nitrogen, and incubated overnight on a shaker at room temperature. Hydrolyzed samples were neutralized with formic acid and cleaned up using solid phase extraction (60 mg MAX SPE columns, Oasis, Waters). Cholesterol and cholesterol oxidation products were extracted with n-hexane during the SPE process and dried and reconstituted in 200 μL of methanol. The final elute (hexane/ethanol/acetic acid mix) of the SPE was extracted, dried, and reconstituted in 200 μL of methanol for the analysis of fatty acids, isoprostanooids, and lipoxigenase (LOX)-derived lipid mediators.31

2.5. Measurement of Polyunsaturated Fatty Acids, Isoprostanooids, and LOX-Derived Lipid Mediators. Polyunsaturated fatty acids (AA, EPA, and DHA), isoprostanooids (F1t-isoP, F2t-isoP, and F3t-NeuroPs), and LOX-derived lipid mediators (5-, 12-, 15-HETEs, and resolvin D1) were quantified according to Leung et al. method using liquid chromatography tandem mass spectrometry (LC-MS/MS). Briefly, a Kinetex HILIC column (30 x 3 mm, 2.6 μm, Phenomenex, USA) was connected to a 1290 Infinity LC system (Agilent Technologies, USA) and maintained at room temperature. The mobile phases were 0.1% acetic acid in water (A) and 0.1% acetic acid in methanol (B). A stepwise gradient elution from 60% B for 5 min,
followed by increasing to 70% in 1 min, and 100% for final 1 min was used. The flow rate was set to 200 μL/min. A QTrap 3200 triple quadrupole mass spectrometer (Sciex Applied Biosystems, USA) was operated in the electrospray ionization (ESI) negative mode with a source temperature of 500 °C and capillary temperature of 250 °C. The collision energy was optimized to maximize the ion currents of the precursor to product ion dissociation. The analytes were detected by MS/MS using multiple reactions monitoring (MRM). Quantitation of each analyte was achieved by relating the peak area with its corresponding internal standard peak. Where available, a heavy labeled isotope was used for quantitation, whereas for those without heavy labeled isotopes quantitation was achieved by relating to those deuterated standards of the closest chemical structure. All PUFAs, isoprostanoids, HETEs, and resolvin D1 standards were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA) or synthesized (4). The gas chromatography (GC) machine operated in the electrospray ionization (ESI) negative mode with a QTrap 3200 triple quadrupole mass spectrometer (Sciex Applied Biosystems, USA) with a source temperature of 500 °C and capillary temperature of 250 °C. The mobile phase was a mixture of 5 mM ammonium acetate in 10% methanol (A) and 5 mM ammonium acetate in 85% methanol (B) with a constant 200 μL/min flow rate. The mobile phase ran at a constant of 0% B phase from 0 to 4 min and gradually increased to 100% from 4 to 5 min, and then B, 100% for subsequent 2 min. Sample injection volume was 20 μL. Positive ESI mode was used with a QTrap 3200 triple quadrupole mass spectrometer (Sciex Applied Biosystems, USA) with a source temperature of 400 °C and a capillary temperature of 250 °C. The analytes were detected by MS/MS using MRM and monitoring the transitions as shown in Figure 1. Negative ESI mode was applied with the source temperature set to 750 °C. Quantitation of lipophenols was achieved by relating the peak area with the internal standard. The standards for lipophenols HT-OA, HT-LA, and HT-ALA were synthesized starting from commercially available dimethoxy-hydroxytyrosol and fatty acids (see Supporting Information for the synthesis description). They were fully synthesized and characterized (using NMR analysis) by Institut des Biomolécules Max Mousseron (IBMM, Montpellier, France). The gas chromatography (GC) machine operated in the electrospray ionization (ESI) negative mode with a QTrap 3200 triple quadrupole mass spectrometer (Sciex Applied Biosystems, USA) with a source temperature of 500 °C and capillary temperature of 250 °C. The mobile phase was a mixture of 5 mM ammonium acetate in 10% methanol (A) and 5 mM ammonium acetate in 85% methanol (B) with a constant 200 μL/min flow rate. The mobile phase ran at a constant of 0% B phase from 0 to 4 min and gradually increased to 100% from 4 to 5 min, and then B, 100% for subsequent 2 min. Sample injection volume was 20 μL. Positive ESI mode was used with a QTrap 3200 triple quadrupole mass spectrometer (Sciex Applied Biosystems, USA) with a source temperature of 400 °C and a capillary temperature of 250 °C. The analytes were detected by MS/MS using MRM and monitoring the transitions as shown in Figure 1. Negative ESI mode was applied with the source temperature set to 750 °C. Quantitation of lipophenols was achieved by relating the peak area with the internal standard. The standards for lipophenols HT-OA, HT-LA, and HT-ALA were synthesized starting from commercially available dimethoxy-hydroxytyrosol and fatty acids (see Supporting Information for the synthesis description). They were fully synthesized and characterized (using NMR analysis) by Institut des Biomolécules Max Mousseron (IBMM, Montpellier, France).
Max Mousseron (IBMM, Montpellier, France) and were used for analysis in dietary lipids for the first time (Figure 1). FA were measured by the concentration ratio using their respective deuterated labeled FA.

2.9. Extraction of Dietary Lipids for Total Polyphenol and Antioxidant Activity Measurement. Lard was prepared from fresh pig fat tissues by heating in the oven at 80 °C for 30 min. Crude lard was obtained by centrifugation at top speed for 2 min and subsequently stored at −20 °C. Extraction of polyphenol and total antioxidants was performed according to Ciafardini et al. with modifications. 

Briefly, 1 g of oil sample was mixed vigorously with 1 mL of hexane and 2 mL of methanol/water solution (60:40, v/v) for 1 min. The lower hydro-alcoholic phase was collected after centrifugation at 800 g for 3 min. All extractions were repeated twice, and the acquired hydro-alcoholic phases were pooled together. Residual oil present in extracts was washed with hexane. Final purified extracts were dried under a stream of nitrogen gas placed on a 30 °C heating block. All concentrated extracts were purged with nitrogen and stored at −20 °C and resuspended in 2 mL of absolute ethanol just before use. All centrifugations were performed at room temperature.

2.10. Measurement of Total Antioxidant Capacity and Total Phenolic Compounds in Dietary Oil and Lard. Total antioxidant capacity from dietary oil and lard was accessed via ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) discoloration assay. Potassium persulfate was added into 7 mM ABTS solution and dissolved in water at a final concentration of 2.45 mM. The mixture was incubated in the absence of light for at least 12 h before being diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. Ten microliters of the sample solution or 2.5 mM Trolox standard was added to 1 mL of diluted ABTS solution for absorbance measurements. The absorbance was measured at 0, 1, and 4 min using a UV/vis spectrophotometer (Genesys 5, Spectronics, Sweden). All determinants were carried out in triplicate. Total antioxidant capacity was calculated in reference to the area under the curve of the Trolox standard and oil samples.

Procedure for polyphenol measurement was adapted from Marinova et al. with a slight modification. Briefly, 1 mL of sample solution was added to 5 mL of Folin–Ciocalteu’s phenol reagent (1:10 dilution with water) and incubated for 4 min. Then, 4 mL of 7% sodium carbonate solution was added and was further incubated for 40 min in darkness. The absorbance was measured at 725 nm using a UV/vis spectrophotometer (Genesys 5, Spectronics, Sweden). All determinants were carried out in triplicate. The standard curve of gallic acid was constructed from 0–12 μg/mL, and the results are expressed as mg gallic acid equivalent (GAE)/g of oil.

2.11. Statistical Analysis. All data were analyzed by GraphPad Prism (version 6 for Mac, USA) and reported as mean ± SD. One-way ANOVA or Student’s t test analysis was tested for statistical significance between control and dietary fat groups. P values of 0.05 or less were considered statistically significant.

3. RESULTS

3.1. Carbon Tetrachloride (CCl4) Induced Hepatic Toxicity and Was Unaffected by Dietary Lipids. The degree of hepatic injury with or without CCl4 in the rodents with different lipid diets was determined by serum ALT and AST activity. Serum ALT and AST were not significantly different in rats fed with different dietary oils in the presence or absence of CCl4 as compared to the control (Figure 2). Nevertheless, the elevation of AST and ALT in CCl4 treated rats confirmed the effect of CCl4 in liver injury but was not affected by the different dietary lipid suppletations.

3.2. Hepatic Histology Displayed Less Modification by EVOO. Hepatic histology with CCl4 treatment in the rodents supplemented with all dietary lipids remarkably showed the formation of adipose vesicles and fibrous septa. In particular, corn oil and lard supplementation were the two most prominent effectors. In contrast, EVOO supplemented rats showed less adipose vesicles and fibrous septa development than those receiving the corn oil or lard diet (Figure 3).
EVOO diet but not RvD1 compared to other dietary lipids and control (Figure 5A). In the presence of CCl₄, the presence of EVOO in the diet consistently showed a lower amount of 5-, 12-, and 15-HETEs productions compared to that of the control (Figure 5B), whereas RvD1 was reduced by EVOO and corn oil. These observations indicate that EVOO could potentially have a strong antioxidant property even under acute and severe oxidative stress.

3.5. EVOO Modulated Cholesterol and COPs Generation.

Elevated cholesterol level was observed in rats fed with all dietary lipids, and it is likely due to the high lipid diet (20%) given to the rats. However, EVOO significantly lowered the cholesterol production in CCl₄ challenged rats, suggesting that EVOO might be able to reverse the cholesterol overproduction or deposition in the liver in the presence of CCl₄. Supplementation of EVOO also reduced 7β-OH 7-ketochol in the presence and absence of CCl₄ but not 7β-OH (Figures 6A and B).

The evaluation of liver COPs in this study further substantiates EVOO as being a strong antioxidant.

3.6. EVOO Had High Total Phenolic Content and Strong Antioxidant Capacity.

The potent inhibition of producing oxidized lipid products under CCl₄ injury by EVOO led us to investigate the total phenolic content and antioxidant capacity of the actual dietary oils that we used. Figure 7A and B illustrates that EVOO possessed the highest concentrations of total phenolic content and antioxidant capacity when compared to that of corn oil and lard. Intriguingly, lard contains a higher amount of total phenolic content and antioxidant capacity than corn oil.

Table 1. Concentration of Precursors of Isoprostanooids Measured in Liver Samples before (-CCl₄) and after (+CCl₄) Carbon Tetrachloride Treatment[^1^]

<table>
<thead>
<tr>
<th>Group</th>
<th>AA -CCl₄</th>
<th>EPA -CCl₄</th>
<th>DHA -CCl₄</th>
<th>AA +CCl₄</th>
<th>EPA +CCl₄</th>
<th>DHA +CCl₄</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>410.43 ± 130.62</td>
<td>204.47 ± 49.61</td>
<td>331.66 ± 74.64</td>
<td>78.05 ± 13.73</td>
<td>215.35 ± 54.23</td>
<td>452.82 ± 87.64</td>
</tr>
</tbody>
</table>

[^1^]Values are the mean ± SD (ng/mg tissue), n = 4–6. Significant difference. ^[^1^]p < 0.01 – CCl₄ vs + CCl₄. *p < 0.01 compared to control of respective group. AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Figure 3. Liver biopsy specimen sections stained with Masson’s trichrome (100 X). Cell nuclei were stained in dark brown, cytoplasms were stained in red, and collagen fibers were stained in blue. Adipose vesicles were denoted with yellow arrows and fibrous septa in green arrows. EVOO, extra virgin olive oil; CCl₄, carbon tetrachloride.

Figure 4. Effect of nonenzymatic lipid peroxidation after dietary oils and lard supplementation (A) and treatment with carbon tetrachloride (CCl₄) (B). Values are the mean ± SD, n = 4–6 rats. The oxidized metabolites are annotated as F₂-IsoPs, 15-F₂-t-isoprostanes from AA; F₃-IsoPs, 15-F₃-t-isoprostanes from EPA; and F₃-NeuroPs, 4(RS)-4-F₄-neuroprostanes from DHA. EVOO: extra virgin olive oil. *p < 0.05 and **p < 0.01 compared to the control.
3.7. High Amounts of Hydroxytyrosol Conjugated FA Are Present in EVOO. Despite the high total phenolic compounds and strong antioxidant capacity in vitro, the bioavailability of EVOO phenolic compounds in humans is extremely poor. Hence, to better understand the potent antioxidant property in vivo, we investigated the proportion of FA (Figure 8A) and the amount of lipophenols (HT-FA) (Figure 8B) in EVOO and OO with respect to CO. Our data (Figure 8A) showed that the major FA profile of both EVOO and OO are similar but different from that of corn oil. Expectedly, OA contributed as the major FA present in EVOO and OO, whereas LA was the main component in CO. Furthermore, high levels of HT-OA, HT-LA, and HT-ALA were found in EVOO and olive oil (Figure 8B) but absent in corn oil. HT-LA in EVOO had the highest concentration among those evaluated, and the concentration of total HT-FA in EVOO was approximately four times more than OO. This suggests that the high content of lipophenols in EVOO and to a lesser extent in OO might increase the bioavailability of hydroxytyrosol in vivo by conjugating with the FA present.

4. DISCUSSION

This study clearly demonstrates the bioactivity of EVOO in the liver. Foremost, it is able to reduce oxidative stress even when challenged by CCl₄. The measurement of isoprostanes, particularly F₂−IsoPs, is often used to unveil the in vivo status of oxidative stress, in which elevated plasma was observed in male and female subjects with NALFD, and the reduction by EVOO in this study indicates a protection mechanism of the liver that may be attributed to lipophenols (HT-FA). A recent study showed the consumption of a Mediterranean diet rich in EVOO for one year reduced urinary F₂−IsoPs in females with metabolic syndrome.
leads primarily to adipophilin protein causes the channelling of fatty acids and modulation of hepatic adipophilin. Down-regulation increased nonesterified fatty acid production by EVOO by the inhibition of Δ⁵-desaturase by gene transcription regulated by omega-3 and Δ⁶-desaturase and lead to competition for per oxidation by EVOO by the formation of AA by the inhibition of Δ⁵-desaturase by gene transcription regulated by omega-3 and Δ⁶-desaturase. It is postulated that the inverse relationship may be attributed to the OA suppression of Δ⁵-desaturase and Δ⁶-desaturase, together with elongase-5 to reduce the formation of AA. Moreover, there is a possibility that AA might suppress the formation of AA by the inhibition of Δ⁵-desaturase by gene transcription regulated by omega-3 and omega-6 PUFA. Further, our findings may be related to increased nonesterified fatty acid production by EVOO by the modulation of hepatic adipophilin. Down-regulation of adipophilin protein causes the channelling of fatty acids and leads primarily to β-oxidation.

It has been recently reported that dietary long chain PUFA with EVOO diet reduced liver steatosis induced by a high fat diet. The report also indicated the suppression of oxidative stress and inflammation by the same diet. However, the study determined an approximate value of TBARS and F₂−IsoPs for oxidative stress using the kit. Also, F₂−IsoPs is only related to AA. Our study is the first to measure multiple oxygenated lipid mediators related to oxidative stress including F₂−IsoPs and F₂−NeuroPs from EPA and DHA, respectively, in the liver with or without CCl₄ challenge and different lipid diets. EVOO was the only dietary lipid in the study to reduce these lipid mediators. Whether this decrease is beneficial or damaging is not clear as major reports focused on the release of these lipid mediators as biomarkers of oxidative stress associated with the brain or the effect of a fish oil diet; both are concentrated in EPA and DHA. However, endogenous elevation of F₂−IsoPs after fish oil supplementation attenuated inflammation in mice, whereas increased F₂−NeuroPs were shown in the plasma of neuro-degenerative disease patients. On the contrary, recent studies indicate that F₂−NeuroPs in LDL−/− mouse livers was associated with lower risk of atherosclerosis, and an in vitro and in vivo study showed F₂−NeuroPs to have antiarrhythmic properties.

Hydroxyeicosatetraenoic acids (HETEs) are oxidized end products nonenzymatically and enzymatically derived from AA. The substrate AA is transformed into 5-, 12-, and 15-HETEs by 5-, 12-, and 15-lipoxygenases (LOX), respectively, or by the action of free radicals. The expressions of 5- and 12-LOX but not 15-LOX have been implicated in carcinogenesis. The forming of 5- and 12-HETEs, are also known to aid in cancer cell proliferations and tumor development, and induce inflammation. In contrast, 15-LOX and 15-HETE appear to inhibit cancerous cell growth. Here, the modulation of HETEs by EVOO supplementation has been suppressed consistently in both the absence and presence of CCl₄ injury. This result suggests that the bioactive EVOO has lessened lipid oxidation and generation of LOX-mediated products in rodents. This is in line with previous reports showing the reduced expression of LOX and their downstream metabolites, HETEs, in the presence of phenolic contents from EVOO. For example, the expression of 5-LOX from a leukocyte was inhibited by hydroxytyrosol (HT), oleuropein, caffeic acid, and tyrosol found in virgin olive oil, in which HT had the strongest effect. Similarly, it was also reported that HT suppressed platelet aggregation and the production of 12-HETE.

LDL oxidation (ox-LDL) is associated with the development of atherosclerosis, and dietary EVOO has shown to lower ox-LDL. Notwithstanding, total cholesterol and its oxidized products are important regulators in the lipidome but are often missing in the studies of EVOO in the liver. An earlier study showed that EVOO and other lipid diets increased hepatic cholesterol in ApoC−/− mice and may be accounted for by the type; EVOO is reported to up-regulate hepatic HDL. Similar to PUFA oxidized metabolites, cholesterol oxidized products (COPs) are both enzymatically and nonenzymatically derived. The 7β−OH and 7-ketochol are interchangeable COPs that arise by free radical reactions from cholesterol, whereas 27-OH is produced enzymatically via a cytochrome P450 system. The production of these COPs is generally disadvantageous as they are being pointed to be disruptive in vivo modulators and biomarkers of LDL oxidation. Here, the type of diets provided to the rats did not alter the liberation of specific COPs in both normal and CCl₄-injured rat groups.

Collectively, profiling of oxidized lipid and cholesterol products from dietary oil supplemented rats against hepatotoxicity induced by CCl₄ showed that EVOO, as compared to the control, consistently reduced the levels of most oxidized lipid products derived via enzymatic and nonenzymatic pathways. This suggests that EVOO supplementation is far more superior

![Figure 8. Percentage of fatty acids (FA) (A) and concentration of lipophenols (B) from EVOO, olive oil, and corn oil. All experimental data were repeated three times and reported as the mean (A) or mean ± SD (B). HT, hydroxytyrosol; CO, corn oil; OO, olive oil; EVOO, extra virgin olive oil; OA, oleic acid; LA, linoleic acid; ALA, alpha-linolenic acid; HT-FA, total sum of HT-OA + HT-LA + HT-ALA. Columns (HT-OA, HT-LA, and HT-ALA) sharing differ supercript letters indicate significant difference p < 0.05. **p < 0.001 HT-FA in OO vs HT-FA in EVOO.](image)
in reducing the rate of in vivo oxidation as compared to other dietary lipids. The reduction of in vivo autoxidation by EVOO is mostly recognized to be attributed to the presence of phenolic compounds, instead of its omega-9-rich MUFA content.\textsuperscript{56} Indeed, measurement of total phenolic content and antioxidant capacity showed EVOO to have higher phenolic amount and higher rate of antioxidant capacity consistently than corn oil and lard. In addition, for the first time we identified that HT may exist as a conjugated form in these dietary oils and potentially link covalently with FA through the primary alcohol of the phenolic derivative. The conjugation with FA may facilitate the bioavailability of HT endogenously through catabolic action and hydrolysis of the intestinal microbiota on the HT-FA ligand.\textsuperscript{57} Depending on the polyphenol structure and their flexibility, the molecule should be able to adjust differently in the position of FA and allow the phenolic component to obtain better interaction with the active site of the enzyme during hydrolysis. Moreover, the number of double bonds in the FA chain could alter the flexibility of the lipophenol conjugate and thus influence its conformation. Take note, if the lipophenol structure contains PUFA, peroxidation may be prevented by the presence of the phenolic residue conjugated, thereby increasing the bioavailability and functionality of the fatty acid as well as the polyphenol when metabolized.\textsuperscript{57}

Recently, vanillyl alcohol conjugated to DHA displayed neuroprotective properties in rat primary neurons exposed to amyloid-\(\beta\) oligomers, and the study suggests that the two molecules synergize for the beneficial effects.\textsuperscript{58} Moreover, when chlorogluconol ring structure was conjugated with DHA, it had a cytoprotective effect on the retinal epithelial cells under carbonyl stress.\textsuperscript{58} In addition, it has been shown that treatment of genistein with omega-3 PUFA in macrophages reduced cyclooxygenase A2 and subsequently inflammatory transcription factor nuclear factor \(\kappa\)B\textsuperscript{59} and that omega-3 PUFA and quercetin supplementation down-regulated intestinal inflammation via an antioxidant effect by quercetin in colitis rats.\textsuperscript{60} As a consequence, in this study, we postulate that the presence of phenolic compounds as a conjugated form with FA (lipophenols) and its antioxidant capacity had a greater effect in reducing oxidative stress.

Several reports have shown EVOO to have a protective mechanism for CVD and reduce inflammation and breast cancer,\textsuperscript{1,56,61} but studies related to the liver are scarce. The liver filters and detoxifies harmful substances in the bloodstream with the help of specific enzymes, ALT and AST, when hepatic cells are damaged or destroyed. To date, ALT and AST activities are regarded as reliable and sensitive markers for liver damage. Intoxication of CCl\(_4\) in animals of different species is a well-known model for inducing acute and chronic liver injury by tetrachloride; CO, corn oil; COPs, cholesterol oxidation products; CHD, coronary heart disease; CVD, cardiovascular disease; DHA, docosahexaenoic acid; DNP, 2,4-dinitrophenyl-hydrazine; EPA, eicosapentaenoic acid; ESI, electrospray ionization; EVOO, extra virgin olive oil; F\(_2\)-IsoP, 15-F\(_2\)-isoprostane; F\(_3\)-IsoP, 15-F\(_3\)-isoprostane; F\(_4\)-NeuroP, 4(\(RS\))-4-F\(_4\)-neuroprostane; FA, fatty acid; HBV, hepatitis virus B; HCV, hepatitis virus C; HETEs, hydroxycicosatetraenoic acids; HT, hydroxytyrosol; HT-ALA, hydroxytyrosol conjugated to alpha-linolenic acid; HT-FA, hydroxytyrosol conjugated fatty acid; HT-LA, hydroxytyrosol conjugated to linoleic acid; HT-OA, hydroxytyrosol conjugated to oleic acid; LA, linoleic acid; LC-MS/MS, liquid chromatography tandem mass spectrometry; LDL, low-density lipoprotein; LOX, lipoxygenase; MRM, multiple reactions monitoring; MUFa, monounsaturated fatty acid; NAFLD, nonalcoholic fatty liver disease; OA, oleic acid;
REFERENCE


