CHEMICAL ENGINEERING TRANSACTIONS
VOL. 49, 2016
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Enrico Bardone, Marco Bravi, Tajalli Keshavarz

ISBN 978-88-95608-40-2; **ISSN** 2283-9216
DOI: 10.3303/CET1649080

Antioxidant and Hepatoprotective Potentials of Phenolic Compounds from Olive Pomace

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Olive pomace, a heterogeneous solid waste from olive oil production, is one of the most widespread agro-industrial by-products in Mediterranean area. The amount of organic compounds with high chemical and biological oxygen demands present in olive pomace stresses the related industries to adapt adequate treatments for this by-product. Being rich in bioactive molecules, it could be considered a low-cost and renewable source of high added value compounds, such as polyphenols. Dietary plant polyphenols are the subject of increasing scientific interest because of their possible beneficial effects on human health. Recent studies suggested that action of polyphenols goes beyond the modulation of oxidative stress and can improve several physiopathological conditions. In the present study, we investigated the possible effects of polyphenols extracted from olive pomace (PEOP) and of the main single phenolic compounds present in the extract (tyrosol, apigenin and oleuropein) in protecting hepatocytes against excess fat and oxidative stress. PEOP were extracted using a high pressure- temperature agitated reactor (25 bar, 180 °C for 90 min). A mixture of ethanol:water (50:50 v/v) was used as extraction solvents resulting to total polyphenol concentration of 5.77 $mg_{Caffeic\ Acid\ Equivalent}$/mL. In order to test the biological effects of the extract we used rat hepatoma FaO cells exposed to a mixture of oleate/palmitate (2:1 molar ratio) for 3 h which represent a reliable *in vitro* model for hepatic steatosis. After lipid-loading, hepatic cells were incubated for 24 h in the absence or in the presence of 10 µg/mL tyrosol, 13 µg/mL apigenin or 50 µg/mL oleuropein. Content of intra- and extra-cellular triglycerides (TGs), and oxidative stress-related parameters were evaluated. The preliminary results showed that PEOP ameliorated lipid accumulation and lipid-dependent oxidative unbalance showing their potential applications as therapeutic agents.

1. Introduction

Polyphenols (PP) constitute the active substances found in many medicinal plants, and range from simple molecules to complex structures (Munin and Edwards-Lévy, 2011). PP are typical micronutrients in our diet, and their role in the prevention of several diseases such as cancer, cardiovascular and degenerative diseases is emerging (Palmieri et al., 2012). All PP show the presence of one or more aromatic rings with one or multiple hydroxyl group as substituents, and they can exist in free form (monomeric or polymeric), complexed to other molecules, such as carbohydrates, lipids, proteins or to other polyphenols. The antioxidant activity is the most relevant biological activity of PP in living systems, and it represents the defence against reactive oxygen species (ROS), such as superoxide radical ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), singlet oxygen (O_2) or peroxyl radicals ($RO_2^{\cdot}$), which can be generated both by exogenous chemicals or endogenous metabolic processes (Georgetti et al., 2008). Due to their antioxidant activity PP play a wide range of biological functions, depending on the biological system or human organ with which they interact (Han et al., 2007; Yoshimura, 2014). In addition to having antioxidant properties, PP have several other specific biological actions that are as yet poorly understood; among them, several authors reported their hepatoprotective potential (Itoh et al., 2010; Poudyal et al., 2010). Ectopic lipid accumulation in the liver (steatosis) is the first step of non-alcoholic fatty liver disease (NAFLD), the most common liver disease in the Western world, where it represents the main link between obesity and its comorbidities such as, type 2

Please cite this article as: Vergani L., Vecchione G., Baldini F., Voci A., Ferrari P.F., Aliakbarian B., Casazza A.A., Perego P., 2016, Antioxidant and hepatoprotective potentials of phenolic compounds from olive pomace, Chemical Engineering Transactions, 49, 475-480
DOI: 10.3303/CET1649080

diabetes mellitus and cardiovascular disease. In the present study, both single PP as well as polyphenols extracted from olive pomace (PEOP) were investigated as potential hepatoprotective agents which could be employed against a wide spectrum of liver diseases.

2. Materials and Methods

2.1 Chemicals

All chemicals, unless otherwise indicated, were of analytical grade and were supplied by Sigma-Aldrich Corp. (Milan, Italy).

2.2 Raw material preparation and polyphenols extraction

Olive pomace of Taggiasca cultivar from a three-phase oil extraction decanter was supplied by an Italian olive oil production plant in the Liguria region, and stored at -20 °C prior to analysis. Olive pomace was washed with *n*-hexane to remove the residual oil, dried in an oven at 60°C for about 48 hours until a constant moisture content was achieved and ground using a laboratory mixer. The extractions of phenolic compounds from olive pomace were performed following the methodology described by Aliakbarian et al. (2011), using a high pressure and temperature (HPTE) reactor (model 4560, PARR Instrument Company, Moline, USA). The extraction was performed at 25 bar and 180°C for 90 min in inert atmosphere (nitrogen) using a solid:liquid ratio of 1:10 (5 g of dried olive pomace and 50 mL of a solution). Mixture of ethanol/water (50:50 v/v) was used as solvent. The polyphenolic extract (PEOP) was centrifuged by an ALC PK131 centrifuge (Alberta, Canada) at 6.000 x*g* for 10 min and filtered through a 0.22 µm syringe filter (Sartorius Stedim Biotech GmbH, Göttingen, Germany) and stored at 4°C prior to experiments.

2.3 Total polyphenolic content determination

Total polyphenolic content (TPC) was evaluated using the Folin-Ciocalteu assay (Gutfinger, 1981). Briefly, 0.2 mL of sample and 0.5 mL of Folin-Ciocalteu reagent were added to 4.8 mL of deionized water; after mixing, 1 mL of a saturated Na_2CO_3 solution and deionized water until a final volume of 10 mL were added. Solutions were mixed and left at room temperature in dark conditions for 60 min. TPC was determined by UV-vis spectrophotometer (PerkinElmer, Milan, Italy) at a wavelength of 725 nm. TPC was expressed as milligrams of caffeic acid equivalents (CAE) per gram of dried powder (DP) (mg_{CAE}/g_{DP}).

2.4 Cell Treatments

Rat hepatoma FaO cells (ECCC, Sigma-Aldrich Corp) are a liver cell line maintaining hepatocyte-specific markers (Lauris et al.,1986). Cells were grown as monolayer in a humidified atmosphere containing 5% CO_2 at 37°C in Coon's modified Ham's F12 medium supplemented with 10% foetal bovine serum (FBS). For treatments, cells were grown until 80% confluence, then incubated overnight in high-glucose serum-free medium with 0.25% bovine serum albumin (BSA). Steatosis was induced by exposing cells to oleate/palmitate mixture (2:1 molar ratio, final concentration 0.75 mM) for 3 h. Thereafter, 'steatotic' cells were incubated for 24 h in the absence or in the presence of single ~~(~~PP~~)~~ (10 µg/mL tyrosol, 13 µg/mL apigenin or 50 µg/mL oleuropein) or, alternatively, in the presence of PEOP at two different concentrations, 0.05 and 0.1 mg_{CAE}/mL. Stock solution of PEOP 10 mM in ethanol/water was diluted with the culture medium to the working concentration.

2.5 Cell viability assay

Viability of FaO cells upon the different treatments was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Wang et al., 1996). Experiments were performed in six replicates in 96-well flat-bottomed culture plates (Sarstedt, Germany). About 2000 cells/well were seeded and, after the treatments, 0.02 ml/well of MTT solution (5 mg/mL in phosphate buffered saline-PBS) was added to the medium and incubated at 37°C for 3 h. After removing the MTT solution, 0.2 ml/well of isopropanol acidified was added, and after agitation for 10 min at room temperature, absorbance at 570 nm was determined in a Microplate Reader on a Varian Cary50 spectrophotometer (Agilent, Milan, Italy).

2.6 Quantification of triglycerides

At the end of the treatments, FaO cells were scraped and centrifuged at 14,000 x*g* for 3 min. After cell lysis, lipids were extracted in chloroform/methanol (2:1) then chloroform was evaporated (Grasselli et al., 2011). In each extract, TG content was determined by spectrophotometric analysis using the 'Triglycerides liquid' kit

(Sentinel, Milan, Italy). Values were normalized for the protein content determined by the bicinchoninic acid (BCA) method using BSA as a standard (Wiechelman et al., 1988). Data are expressed as percent TG content relative to controls.

2.7 Lipid peroxidation measurement

Lipid peroxidation was determined through the thiobarbituric acid reactive substances (TBARS) assay based on the reaction of malondialdehyde (MDA; 1,1,3,3-tetramethoxypropane) with thiobarbituric acid (TBA) (Iguchi et al., 1993). Briefly, 2 volumes of TBA solution (0.375% (w/v) TBA, 15% (w/v) trichloroacetic acid, 0.25 N HCl) were added to 1 volume of the cell suspension and incubated for 45 min at 95°C. Then, 1 volume of N-butanol was added and the organic phase was read at 532 nm using a Varian Cary50 spectrophotometer. The MDA level was expressed as pmol MDA/ml x mg of sample.

3. Results and discussion

3.1. Total polyphenol content of the extracts

For the extraction of the polyphenols different ethanol/water ratios were tested in order to obtain an extract with high antioxidant properties. TP yields, expressed as mg_{CAE} per gram of dried matter (mg_{CAE}/g_{DM}) of extracts are shown in Table 1.

Table 1: Yield of total polyphenols (TP) extracted from olive pomace using solutions at different ethanol/water ratio as solvent.

Ethanol:water ratio	TP yield (mg_{CAE}/g_{DM})
0:100	20.7±1.1
25:75	32.8±0.2
50:50	57.7±0.5
75:25	59.5±0.9
100:0	23.8±0.9

As shown, the addition of ethanol from 0 to 75 % (v/v) in the solvent mixture increases approximately 3 times the TP yield. This can be explained considering that a higher concentration of ethanol leads to a better solubility of phenolic compounds and a higher pressure in the reactor, which can enhance the matrix disruption. A further increase in ethanol content led to a decrease in the phenolic content of the extract, indicating that a fraction of water is necessary to obtain high yields. As can be seen, the highest TP yields were obtained with ethanol/water ratios of 50:50 and 75:25. On the basis of these results, an extract of ethanol:water of 50:50 (v/v) was used for the biological evaluation tests.

3.2. Effects of polyphenols on lipid accumulation and cell viability

To mimic hepatic steatosis, FaO cells were overloaded of lipids by exposure to an oleate/palmitate mixture for 3 h, then cells were treated with single PP or PEOP for 24 h. The intracellular TG accumulation was quantified in control (C) and ‘steatotic’ cells incubated in the absence (OP) or in the presence of the single polyphenols Apigenin (OP+A), Tyrosol (OP+T), Oleuropein (OP+O) (Figure 1). Results are expressed as percentage values with respect to control. Significant differences *p* are denoted by symbols on bars.TG content markedly increased in ‘steatotic ‘cells with respect to C (+131%; $p \leq 0.001$). Incubation of ‘steatotic’ cells with the single PP led to different effects in terms of lipid accumulation. While tyrosol and oleuropein significantly reduced the TG content of -76% ($p \leq 0.001$) and -73% ($p \leq 0.001$), respectively, compared to ‘steatotic’ cells, no significant effects were observed with apigenin. The exposure of ‘steatotic’ cells to PEOP (Figure 1) led to a significant reduction in the TG content (-74%; $p \leq 0.01$) only for the lower concentration of 0.05 mg_{CAE}/mL. In control cells, the lipid content was not affected by both single PP and PEOP (data not shown). Moreover, in our conditions, OP treatment did not show significance toxicity due to the higher proportion of palmitate with respect to oleate (2:1 oleate/palmitate) that has minor toxic and apoptotic effects (Gómez-Lechón et al., 2007), and also, both PP and PEOP did not affect significantly cell viability (data not shown).

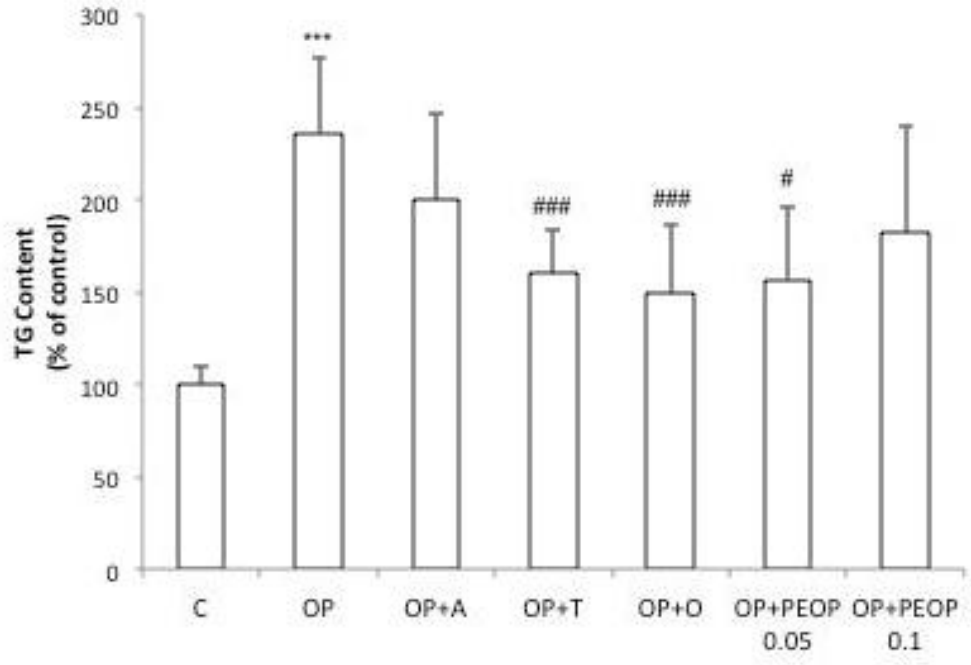

*Figure 1: TG quantification in cells exposed to OP (0.75 mM) alone or combined with the single polyphenols or PEOP for 24 h. ***p≤0.001 all treatments vs C; ##p≤0.01 and #p≤0.05 OP vs PEOP.### p≤0.001 OP vs polyphenols.*

3.3. Effects of polyphenols on oxidative stress

Lipid peroxidation is one of the most widely used indicators of ROS-induced oxidative stress. Here, we assessed lipid peroxidation by measuring the MDA level through the TBARS assay (Figure 2). Data are expressed as percentage values with respect to controls. In 'steatotic' cells, the MDA level was increased of +122% ($p \leq 0.01$) with respect to C. The incubation of 'steatotic' cells with Apigenin (OP+A) or Oleuropein (OP+O) reduced the MDA level of -74% ($p \leq 0.05$) and -67% ($p \leq 0.05$), respectively, compared to 'steatotic' cells. By contrast, no significant effects were observed with Tyrosol. In control cells, the MDA level was not affected by the single PP (data not shown). The exposure of 'steatotic' cells to PEOP extract significantly reduced the MDA level of -102% ($p \leq 0.01$) only for the higher concentration of 0.1 mg_{CAE}/mL.

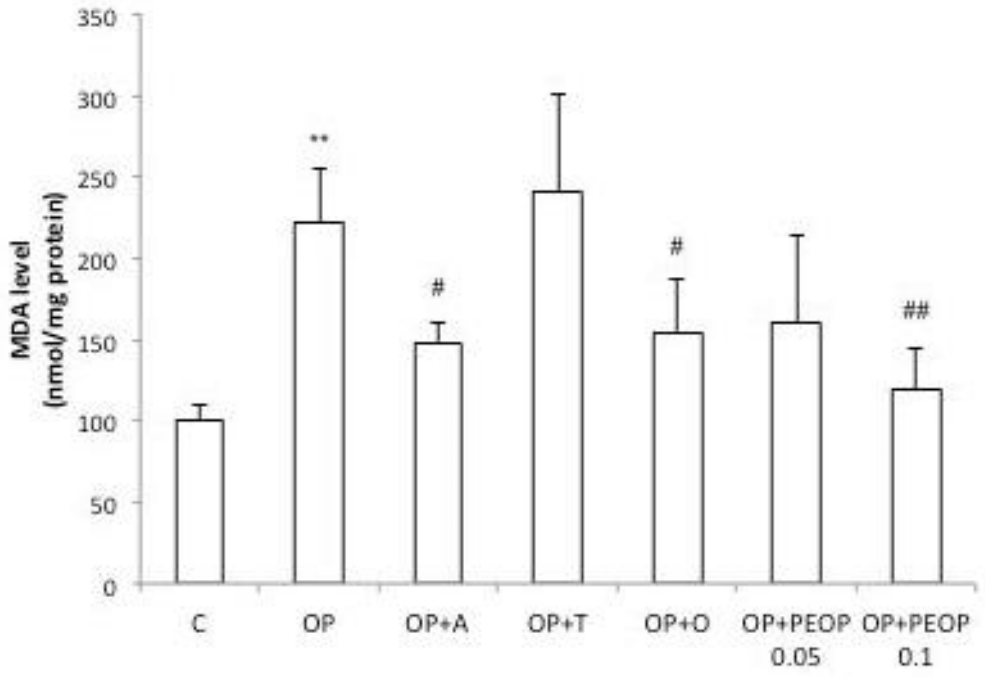

*Figure 2: MDA levels (% of control) in cells exposed to OP (0.75 mM) alone or combined with the single polyphenols or PEOP for 24 h **p≤0.01 all treatments vs C; #p≤0.05 OP vs polyphenols, ##p≤0.01 OP vs PEOP extract.*

4. Conclusions

In the last decade, there was increasing interest in the potential health benefits of dietary plant polyphenols as antioxidants. Moreover, several recent studies suggested possible hepatoprotective actions of polyphenols. In the present study, we used an '*in vitro'* model of hepatic steatosis to test the possible antisteatotic and antioxidant properties of a polyphenol mixture extracted from olive pomace. For comparison, the effects of the main single phenolic compounds present in the extract (tyrosol, apigenin, and oleuropein) were assessed. Our results show that the exposure of rat hepatoma FaO cells to both single PP and to PEOP significantly reduced both the TG accumulation and the lipid peroxidation without significantly affect cell viability. The anti-steatotic effects of PP both as single agents and as extract may depend on reduction of lipogenesis and/or stimulation of lipolysis (mainly fatty acid β-oxidation). It has to be noted that whereas the antioxidant effect was more marked for the higher PEOP concentration, the anti-steatotic effect was more evident for the lower dose. However, a non-monotonic response to increasing concentrations of a substance has been widely described in endocrinology. Previous studies reported that commonly consumed polyphenols, such as those used here, have a more or less pronounced effect on fat accumulation, but the novelty of our approach is to use a PP mixture. Thanks to all these positive properties, PEOP may lead to a novel nutraceutical formulation, in which the nutritive characteristics of food can be enriched with the health benefits related to polyphenols consumption.

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