

Protective effect of olive mill wastewater against lipid metabolism perturbation induced in cafeteria diet fed rats

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Abstract: The biological activities of phenolic compounds from olive mill wastewater (OMWW) have been extensively studied. However, the bioactivity of raw OMWW has not been yet well demonstrated in vivo. The objective of this study was to evaluate the effect of oral administration of OMWW on cafeteria diet induced metabolic disorders in rats. Our results showed that within a 13-week treatment period, the cafeteria diet induced an increase in glucose and triglycerides levels. The levels of glucose, triglycerides and the total cholesterol were significantly decreased after supplementation with OMWW ($p < 0.05$). To evaluate the toxicity effect of OMWW, the results concerning the blood parameters of the renal and liver function (urea, creatinine, uric acid, bilirubin, total protein and transaminases) showed that there are not difference between the control and the OMWW supplementation groups. The liver histology from rats fed with cafeteria diet showed a steatosis which is characterized by lipid droplet accumulation in the hepatocytes cytoplasm. In OMWW supplemented groups, the liver tissue showed a structure similar to the control group. HPLC analysis of OMWW revealed the major presence of a phenolic acid which is hydroxytyrosol. In conclusion, our study suggests that OMWW brought real health benefits by protecting liver tissue and modulating some biological parameters such as lipid parameters.

I. Introduction

Obesity is one of the most important risk factors contributing to cardiovascular diseases [1]. In the Mediterranean area, the incidence of heart disease is lower due to a diet that is based mainly on fruits, vegetables and olive oil fat [2]. Olive oil is obtained by pressing the olive fruit, which contains a variety of phenolic compounds. During olive oil extraction, large amounts of water are generated and

subsequently discarded. These olive mill wastewaters (OMWW) contain notable amounts of polar phenolic compounds (approximately 53 %) [3], including hydroxytyrosol (HT), tyrosol, catechol, caffeic acid, and *para*-coumaric acid, which are major contributors to the environmental toxicity of OMWW [4][5]. These phenolic compounds, which are present in general in olives, olive oil and olive byproducts [6], are endowed with several biological activities such as antioxidant properties [7][8], inhibit platelet aggregation,

lipoxygenases and eicosanoid production [9][10]. However, these phenolic compounds also have hypoglycemic and hypolipidemic properties. Indeed Hamden et al.[11][12] reported that the administration of hydroxytyrosol extracted from olive mill waste decreases plasma glucose and cholesterol levels in diabetic rats. In addition, Cao et al.[13] show that HT could prevent high-fat-diet (HFD) induced obesity, hyperglycemia and hyperlipidemia in C57BL/6 J mice. In fact, HT is an effective compound that exerts a protective effect against dyslipidemia and hyperglycemia observed in several pathologies such as obesity, diabetes and metabolic syndrome. The extraction methods of polyphenols from OMWW are expensive and now a day, data concerning the effects of raw OMWW *in vivo* has not been reported yet. Therefore, in the present work, the hypolipidemic effects of OMWW on plasma lipid levels and the histological structure of liver tissue were analyzed using a model of rats who have received a cafeteria diet. In addition, the probably toxic effects of OMWW on the renal and liver function were also investigated.

II. Materials and methods

II.1. Preparation of OMWW

Fresh olive mill wastewater was supplied by a discontinuous olive milling plant from a cooperative in Kabylia region (Algeria). This sample was collected in the mid olive harvesting season (January 2015) and conserved at 4 °C. In our experiment, samples have not been treated with the exception of a decantation in order to eliminate a maximum of fat. The partial characterization of olive mill waste consists in determining the dry matter content, pH, total carbohydrates, lipids and total nitrogen. The pH was measured using a pH meter. The total dry matter was determined after drying at 105 °C. The total sugars was measured using the Dubois method [14]. The OMWW fats content was estimated following an extraction with hexane solvent. Total Nitrogen (TN) was determined following the Kjeldahl method (Table 1).

Table 1. Parameters of olive mill wastewater

Parameters	OMWW
pH (20 °C)	4.65
Dry matter (g/L)	50.5 ± 1.32
Total sugars (g/L)	5.16 ± 0.56
Lipids (g/L)	0.6 ± 0.04
Total Nitrogen (TN) (mg/L)	431 ± 27

II.2. HPLC analysis

A reverse phase high performance liquid chromatography method was developed to identify and quantify the major phenolic compounds contained in olive mill wastewater. Concentrations were calculated based on the peak areas compared to those of the authentic standards. The HPLC chain used is of the "Agilent technologies series 1260" type. It includes a binary pump, a diode array UV detector, an automatic injector and a thermostatted column compartment. The separation is carried out on a C18 column (250 mm in length x 4.6 mm in diameter x 3.5 µm as particle size). The mobile phase used is composed of two solutions: 0.1% acetic acid in water (A) and 100% acetonitrile (B) with a flow rate of 0.5 ml / min. Phenolic compounds were identified by comparison with commercial standards and by verification of their corresponding absorption spectra.

II.3. Animals and treatment

All the experiments were carried out according to the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA), following approval by the Institutional Animal Care Committee of the Algerian Higher Education and Scientific Research. The permits and ethical rules were achieved according to the Executive Decree n_10-90 completing the Executive Decree n_04-82 of the Algerian Government, establishing the terms and approval modalities of animal welfare in animal facilities. This was also, recently supported by the USTHB university ethical committee of the "Algerian Association of Animal Experimentation Sciences" AASEA(AgreementNumber45/DGLPAG/DVA.SD A.14).

Thirty male Wistar rats weighing between 220 and 250 g were purchased from Pasteur Institute (Algiers). The animals were individually housed in cages at a 25 °C controlled temperature and 12 h light and darkness alternating periods. The rats were randomly divided into four groups. Group 1 (Control group n= 6) was fed with a standard diet (SD). Group 2 (n= 9) was given a standard diet with incorporation of raw OMWW (SDO) in drinking water (dilution 11 times in water). Groups 3 (n= 6) was given a cafeteria diet (CD) and 4 group (n= 9) received a cafeteria diet with OMWW in drinking water (CDO). The duration of the treatment was 13 weeks. The body weight was measured every week. At the end of the experimental period, the rats were sacrificed by decapitation. Plasma samples were collected and stored at -80 °C until analysis.

II.4. Diet composition

The cafeteria diet used in our study is a hyper-caloric and hyper-lipidic diet. It was composed of 50% standard diet and 50% of a mixture of

products made locally and containing: cookies - pâté - cheese - chips - peanut - chocolate, in proportions of: 2: 2: 2: 1: 1: 1. The standard regime is manufactured by SARL local production (Cattle Feed Agglomerates, Bouzarea, Algiers, Algeria). The characterization of these diets consists in carrying out assays of lipids, sugars and proteins according to the industrial processes. The characteristics of the used diet are presented in Table 2.

Table 2. Composition of the experimental diets (g/100g diet)

Parameters (g/100g diet)	Standard diet	Cafeteria diet
Sugars	2 g	13.86 g
Lipids	4.53 g	24.34 g
Proteins	19 g	10.18
Energetic value	124.77 Kcal (521.53 KJ)	315.22 Kcal (1317.61 KJ)

II.5. Biochemical analysis

Quantitative estimation of plasma triglyceride (TG), glucose, total cholesterol (TC) levels and high-density lipoproteins (HDL), hepatic enzymes: (AST) and (ALT), total protein, total bilirubin, Uric acid, Urea and creatinine were measured using an automatic biochemistry analyzer Abbot Architect Ci 4100at laboratory Zerrar at Tizi-Ouzou, Algeria.

II.6. Liver histopathological analysis

Three livers were randomly selected from each group for routine light microscopy observation. They were taken freshly and immediately fixed in 10% formalin fluid, embedded in paraffin and serially sectioned at 5µm. These sections were stained with haematoxylin eosin (HE) for routine histological examination. Liver steatosis was considered to assess the histological integrity. Semi-quantitative assessment was performed using scores ranging from 0 to 4 as follows: (1+) represents a 25% loss, (2+) represents a 50% loss, (3+) represents a 75% loss and (4+) represents more than 75% loss [15][16]. The score was determined in 20 randomly selected sections which were examined at 100X magnification.

II.7. Statistical analysis

All data were expressed as means \pm standard deviation (mean \pm SD). Differences between treatments were analyzed by one-way ANOVA, followed by Tukey's post hoc test for multiple comparisons with statistical significance. The software used is minitab18.

III. Results and discussion

III.1. Body growth

During the study period, a regular increase in body growth rate was noticed in cafeteria diet-fed rats (Figure 1). At day of sacrifice, final body weight of the CD group showed a significant increase ($p < 0.05$) compared to the control group (SD). OMWW supplementation in rats of both groups (SDO) and (CDO) induced a no significant decrease in weight.

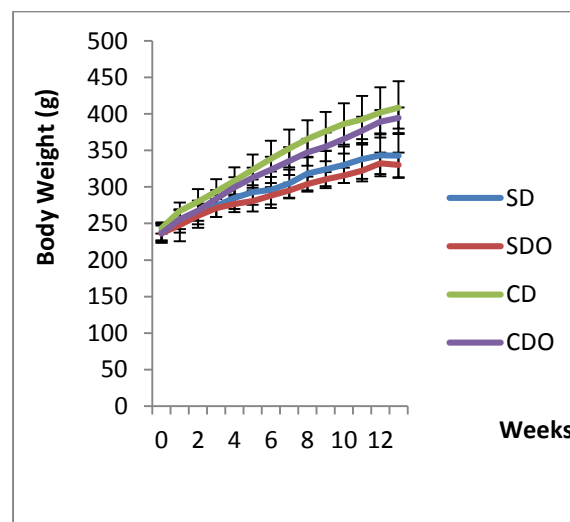


Figure 1. Body weight (g) of male rats during the experimental period. Control group (SD), rats treated with OMWW (SDO), cafeteria diet-fed rats (CD) and cafeteria diet-fed rats treated with OMWW (CDO).

III.2. HPLC analysis

In the present study, we investigated the effect of dietary phenolic compounds from OMWW in rats given a cafeteria diet. Through HPLC analysis (Figure 2), OMWW has showed few phenolic compounds with hydroxytyrosol being the major compound with a concentration of 0.25 g/L with trace amounts of tyrosol. Previous reports showed a predominant presence of hydroxytyrosol in OMWW at important levels (1.225 g/L and 1.433 g/L) [17][18].

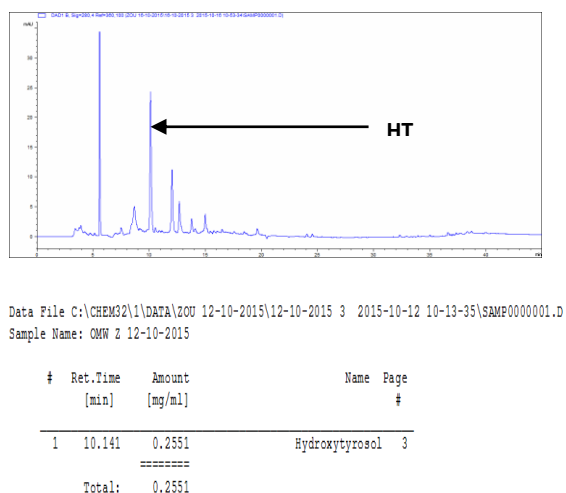


Figure 2. High performance liquid chromatography (HPLC) at 280 nm of raw olive mill wastewater (OMWW). HT: Hydroxytyrosol.

III.3. Plasma parameters

Cafeteria diet induced a perturbation in plasma sugar and lipid parameters. Unlike Cholesterol and High-Density Lipoprotein (HDL), the plasma of triglyceride and glucose levels showed an increase compared to the control group ($p < 0.05$) (Figure 3). Concerning total cholesterol, triglyceride and glucose levels, following supplementation of OMWW to standard and cafeteria diet-fed rats, a significant amelioration was displayed ($P < 0.05$). Similar effect was observed for HDL level but only in CDO group.

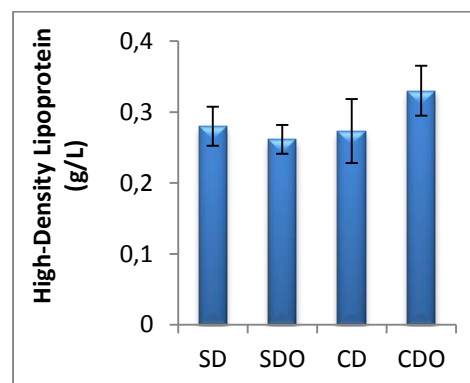
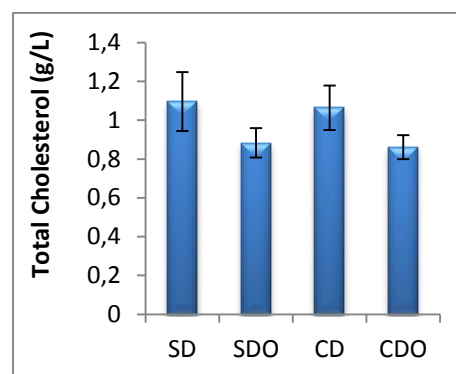
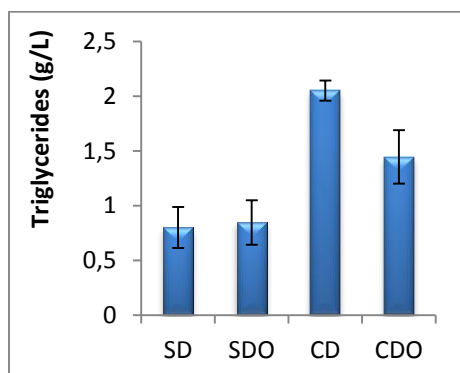
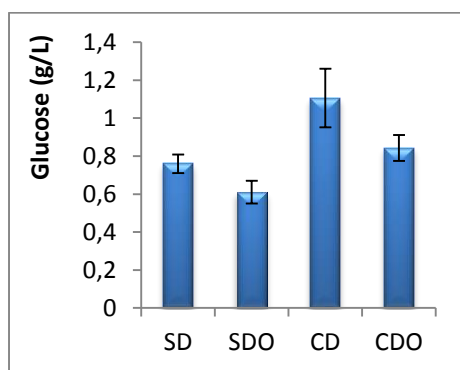


Figure 3. Effect of cafeteria diet and its combination with OMWW on plasma lipid parameters: triglycerides (g/L), cholesterol (g/L), HDL (g/L) and glucose (g/L). Control group (SD), rats treated with OMWW (SDO), cafeteria diet-fed rats (CD) and cafeteria diet-fed rats treated with OMWW (CDO).

For the hypoglycemic effect of OMWW, Hamden *et al.* [11][12] reported that hydroxytyrosol decrease plasma glucose levels in rats. This hypoglycemic effect might be explained by the inhibition of the activity of maltase, lactase and sucrose, the most important carbohydrate enzymes located in antherocytes and catalyze the breakdown of disaccharides into simple sugars, readily available for intestinal absorption [19][20].

The concentration of total cholesterol was significantly reduced by an oral intake of OMWW. Similar results were reported by previous studies but only about OMWW phenols. Indeed, Jemai *et al.* [21] and Fki *et al.* [22][17] have shown that oral administration of hydroxytyrosol or total polyphenols extracted from OMWW to Wistar rats induced a hypocholesterolemic effect by a significant decrease of CT, TG and LDL-C (Low-density lipoprotein) levels and an increase in HDL. Hamden *et al.* [12] reported that purified HT exerts a potential inhibition of intestinal lipase activity. Thus, this inhibitory effect decreases the hydrolysis of dietary triglycerides to monoglycerides and free fatty acids, resulting in a decrease in lipid

absorption. Our results suggest that OMWW has a potent serum lipid-lowering effect in cafeteria diet-fed rats. Indeed, the intake of diluted raw OMWW induced a protective effect against lipid metabolism perturbation.

Concerning transaminase levels, urea, total bilirubin, uric acid, total protein and creatinine, there is no significant difference between the control group (SD) and OMWW-treated (SDO) group (Table 3). However, the levels of transaminases and uric acid decreased significantly for the CD group ($p < 0.05$), probably because the cause is attributed to the cafeteria diet, however the OMWW supplementation (CDO group) seems to improve the levels of these parameters. These results show that OMWW does not affect blood parameters related to renal and hepatic function.

Table 3. Plasma levels of ALT (UI/L), AST (UI/L), total protein (g/l), total bilirubin (mg/l), uric acid (mg/l), urea (g/l) and creatinine (mg/l). controls (SD), treated with OMWW (SDO), CAF-diet (CD) and OMWW with CAF-diet (CDO). Treated (SD, SDO, CDO) vs. control (CD): (a,b,c) $p \leq 0.05$. Treated (SD, SDO) vs. treated (CDO): (d,e) $p \leq 0.05$.

Blood parameters	SD	SDO	CD	CDO
AST (UI/L)	427 \pm 63.83	406.66 \pm 35.75	227.6 \pm 26.50	325.3 \pm 61.86
ALT (UI/L)	125 \pm 05	115.8 \pm 13.16	64.5 \pm 12.06	74.12 \pm 13.27
Total protein (g/L)	91 \pm 3.55	90 \pm 5.44	91.75 \pm 6.39	89.87 \pm 7.39
Total bilirubin (mg/L)	1.2 \pm 0.44	1.71 \pm 0.48	1.25 \pm 0.5	1.37 \pm 0.51
Uric acid (mg/L)	29.25 \pm 1.25	26.66 \pm 3.01	17.5 \pm 1.73	24.5 \pm 3
Urea (g/L)	0.56 \pm 0.07	0.50 \pm 0.04	0.56 \pm 0.04	0.44 \pm 0.02
Creatinine (mg/L)	10.33 \pm 0.57	9.25 \pm 0.5	9.66 \pm 0.57	9.75 \pm 0.88

III.4. Histological study

Light microscopic observation of hepatic tissue within the control (SD) and the OMWW-treated (SDO) group showed normal large polygonal cells with prominent round nuclei and eosinophilic cytoplasm (Figure 3, SD, SDO). Regarding the

cafeteria diet fed group, the liver histopathological observation showed a micro-vesicular steatosis and a leukocytes inflammatory cells infiltration mainly in the portal triad (Figure 4, CD). The liver tissue from cafeteria diet-fed rats supplemented with OMWW appeared similar to those of control rats (Figure 4, CDO).

Histopathological examination of liver tissue supported the biochemical data and shows the powerful activity of OMWW in reducing steatosis induced by cafeteria diet. Similar previous statements indicated that olive oil phenols and hydroxytyrosol able to reduce liver steatosis induced by high cholesterol diet [23][24][25].

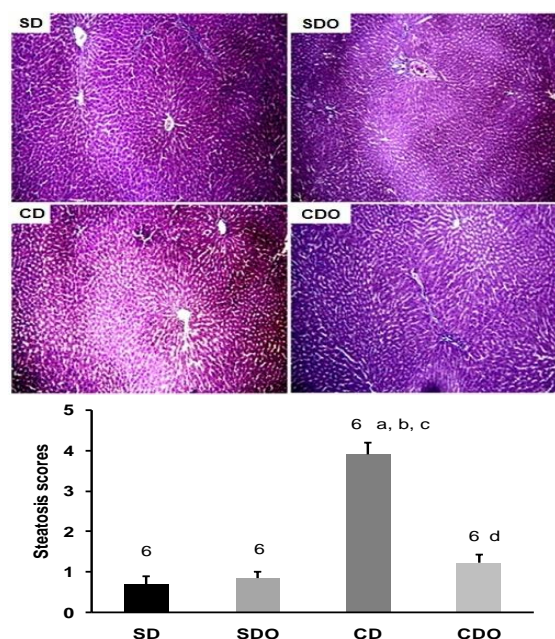


Figure 4. Histological aspect and semi-quantitative scores of liver injury in control and treated groups. HE staining; magnification: 100X. Control group (SD), rats treated with OMWW (SDO), cafeteria diet-fed rats (CD) and cafeteria diet-fed rats treated with OMWW (CDO). a,b,c for statistical comparison (SD, SDO, CDO) vs CD; d for statistical comparison SD vs CDO; $p \leq 0.01$.

Histological data are also in agreement with biochemical findings. Indeed, lipid droplets accumulation was observed in the cytoplasm of hepatocytes from cafeteria diet fed rats. This phenomenon known as microvesicular steatosis has been largely discussed [17].

IV. Conclusion

In summary, the present study demonstrated that OMWW, eventually through its phenolic compounds, has a very pronounced hypolipidemic and hypoglycemic activity and may protect the liver function against cafeteria diet damaging effects. Furthermore, our results showed that raw OMWW had not toxic effects in hepatic and renal functions. These properties are interesting regarding its uses as therapeutic agent in biotechnological applications especially in developing hypocholesterolemic drugs, therefore it can be used as a powerful agent in the prevention of cardiovascular diseases and an anti-obesity drug.

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