Hypoglycemic and antioxidant effects of phenolic extracts and purified hydroxytyrosol from olive mill waste in vitro and in rats

Khaled Hamden a,⁎, Noureddine Allouche b,⁎, Mohamed Damak b, Abdelfattah Elfeki a

aAnimal Ecophysiology, Faculty of Sciences, Sfax, Tunisia
bLaboratory of Chemistry of Natural Products, Faculty of Sciences of Sfax, B.P. 1171, 3000 Sfax, Tunisia

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ABSTRACT

This study aimed to evaluate the effect of phenolic extract and purified hydroxytyrosol (HT) from olive mill waste (OMW) on oxidative stress and hyperglycemia in alloxan-induced diabetic rats. The OMW biophenols were extracted using ethyl acetate. The obtained extract was fractionated by solid phase extraction (SPE) experimentation to generate two fractions: (F1) and (F2). HPLC-UV and HPLC-MS analysis showed that (F1) was made of known OMW monomeric phenolics mainly hydroxytyrosol (HT) while (F2) contained oligomeric and polymeric phenols such as verbascosid and ligstrosid. (HT) was purified from (F1) using silica gel-column chromatography and silica gel-TLC techniques. In incubated pancreas, supplementation of OMW fractions enhanced insulin secretion. The administration of OMW extract fractions (F1) and (F2) as well as purified (HT) in diabetic rats caused a decrease in glucose level in plasma and an increase in renal superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) activities in liver and kidney. Furthermore, a protective action against hepatic and renal toxicity in diabetic rats was clearly observed. Furthermore, a significant decrease in hepatic and renal indices toxicity was observed, i.e. alkalines phosphatases (ALP), aspartate and lactate transaminases (AST and ALT) activities and the thiobarbituric acid-reactive substances (TBARs), total and direct bilirubin, creatinine and urea levels. In addition, (F1), (F2) and especially (HT) decreased triglycerides (TG), total-cholesterol (T-Ch) and higher HDL-cholesterol (HDL-Ch) in serum. These beneficial effects of OMW biophenols were confirmed by histological findings in hepatic, renal and pancreatic tissues of diabetic rats. This study demonstrates for the first time that OMW polyphenols and especially (HT) are efficient in inhibiting hyperglycemia and oxidative stress induced by diabetes and suggests that administration of HT may be helpful in the prevention of diabetic complications associated with oxidative stress.

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1. Introduction

Type 1 diabetes is a metabolic disorder caused by complete or relative insufficiency of insulin secretion [1] and is the main cause of numerous complications related to many diseases. Chronic elevation of blood glucose will eventually lead to tissue damage, with consequent often serious disease. While evidence of tissue damage can be found in many organ and systems [1,2]. Hyperglycemia leads to long-term complications of diabetes, which are the major causes of morbidity and mortality in human populations [3]. Increased free radical generation and oxidative stress are hypothesized to play an important role in the pathogenesis of diabetes and its late complications [3]. In the past few years, polyphenolics substances have received widespread attention because of their potential for preventing some highly prevalent chronic diseases. In fact it has been reported that polyphenols are endowed with interesting biological activities such as anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities [4–6]. In addition, olive mill waste (OMW) biophenols are efficient components for the low incidence of cardiovascular diseases in the Mediterranean area [7]. A hen, olive mill waste is potentially a rich source of a diverse range of biophenols with a wide array of biological activities [7]. The beneficial health effects of OMW have been mainly attributed to its elevated phenols content [8]. Among them, hydroxytyrosol (3,4-dihydroxyphenylethanol) stands out as a compound of high added-value, due to its interesting antioxidant and potential beneficial human health properties [9]. Hydroxytyrosol has various biological activities, such as down-regulation of the immunological response [10], preventing human erythrocytes from oxidative damage induced by hydrogen peroxide [11], anti-inflammatory, antithrombotic, and hypcholesterolemic effects in rats [12–14]. Animal study has shown that administration of 100 mg/kg hydroxytyrosol for 12 days enhanced resistance...
of dissociated brain cells to oxidative stress, as shown by reduced basal and stress-induced lipid peroxidation [13]. Furthermore, a previous report has indicated that hydroxytyrosol is able to cross the blood–brain barrier [14]. The present study describes the bio-phenol extraction, fractionation and identification from OMW. In addition, it deals with the evaluation of hypoglycemic and antioxidant activities of the OMW monomeric and polyphenolic phenols as well as of purified hydroxytyrosol.

2. Materials and methods

2.1. Materials

Fresh olive mill waste (OMW) was supplied by a discontinuous three phase olive processing mill from a cooperative in Sfax (Tunisia). This sample was taken at the middle of the olive harvest season (January 2008) and conserved at 4 °C. Tyrosol, caffeic acid, para-coumaric acid, protocatechuic acid, para-hydroxyphenylacetic acid (PHPA), luteolin, apigenin and rutin were purchased from Sigma–Aldrich. Hydroxytyrosol was purified in our laboratory from OMW as described previously [15]. Oleuropein and ligstrosid were obtained from Extrasynthese (France). All solvents were analytical grade and used without further purification.

2.2. Extraction of phenolic compounds from OMW

Liquid–liquid extraction with ethyl acetate was carried out on OMW (4 l) in a separatory funnel. The mixture (solvent-OMW) was vigorously shaken for 10 min to achieve equilibrium and was then allowed to settle for 30 min. The phases were separated and the extraction was repeated successively four times. All the runs were performed at ambient temperature (20–25 °C). The ethyl acetate combined fractions were dried over anhydrous sodium sulphate and the ethyl acetate was evaporated in a rotary evaporator at 40 °C to yield 47.31 g. The obtained dry residue was used for characterization, quantification and fractionation of phenolic compounds as well as for hydroxytyrosol purification.

2.3. OMW extracts fractionation

Fractionation of OMW phenolic extract was carried out by solid phase extraction (SPE) experimentation. OMW extract (45 g) was subjected to a flash chromatography on reversed-phase (VersaPak™, C18 Cartridge 40 mm × 75 mm) under medium pressure using a gradient mobile phase consisting of H2O–MeCN from 80:20 to 0:100. The flow rate was adjusted to 10 ml/min and 8 ml fractions were collected. These fractions were measured by optical density at 280 nm and the chromatogram (optical density versus fraction number) was represented (data not shown). Two separated peaks were obtained. The corresponding fractions were collected into two sub-fractions: (F1) and (F2) related to the first and the second separated peaks, respectively.

2.4. Hydroxytyrosol purification

For hydroxytyrosol purification, an aliquot (4 g) of the dry first fraction (F1) was chromatographed on a silica gel-column (4 cm × 80 cm) eluted with a solvent gradient: hexane–ethyl acetate–methanol. The chromatographic behavior was followed by TLC analysis using silica gel plates (Merck, 60 F-254). Nine groups of homogeneous fractions were collected. The third group was purified by prep.TLC, eluted with a mixture of chloroform:methanol (9:2, v/v), which afforded one pure compound with Rf = 0.6. This compound was identified as hydroxytyrosol (designated F3) by means of HPLC-UV and HPLC-MS analyses.

2.5. HPLC separation and identification of phenolic compounds

The presence and amount of phenolic compounds in the OMW extract were studied by reversed-phase HPLC analysis using a binary gradient elution. The analysis was performed by reversed-phase HPLC on a Waters autopurification system equipped with a binary pump (Waters 2525), a UV–vis diode array detector (190–600 nm, Waters 2996) and a PL–ELS 1000 ELS detector Polymer Laboratory. The chromatographic separation was achieved on a Kromasil C18 column (250 mm × 4.6 mm, I.D., 5 m, Thermo). Its temperature was maintained at 40 °C. The mobile phase was 0.1% formic acid in water (A) versus 0.1% formic acid in acetonitrile (B) for a total running time of 60 min. The elution conditions were: 0–30 min, 20–50% B; 30–35 min, 50% B; 35–45 min, 50–100% B; 45–55 min, 100% B; 55–60 min, 100–20% B. Finally, the column was subjected to washing and reconditioning steps for 10 min with 20% B. The flow rate was 0.6 ml/min and the injection volume was 50 µl. The main phenolic compounds were identified by comparison with relative retention times and UV spectra of pure standards, when available, or by comparing the relative elution order and UV spectra with those reported in the literature [16,17].

The identity of each peak was confirmed by LC–MS, performed on an Agilent 1100 LC system consisting of degasser, binary pump, auto sampler, and column heater. The column outlet was coupled to an Agilent MSD Ion Trap XCT mass spectrometer equipped with an ESI ion source. Data acquisition and mass spectrometric evaluation was carried out on a personal computer with Data Analysis software (Chemstations). For the chromatographic separation, the same conditions (column, solvent gradient and flow rate), given previously, were used. The following parameters were employed throughout all MS experiments: for electrospray ionisation with positive ion polarity the capillary voltage was set to 3.5 kV, the drying temperature to 350 °C, the nebulizer pressure to 40 psi, and the drying gas flow to 10 l/min. The maximum accumulation time was 50 ms, the scan speed was 26,000 mz −1 s−1 (ultra scan mode) and the fragmentation time was 30 ms. Identification of compounds by LC–MS analysis was carried out by comparing retention times and mass spectra of the unknown peaks to those of standards, when available.

2.6. Animals

Male Wistar rats, with body weights of 180–200 g and bred in the Central Animal House and obtained from the Central Pharmacy, Tunisia, were used in this study. The animals were fed on a pelleted diet (Socco, Sfax, Tunisia) and water ad libitum. The animals were maintained in a controlled environment under standard conditions of temperature and humidity with an alternating light-and-dark cycle. The handling of the animals was approved by the Tunisian Ethical Committee for the care and use of laboratory animals.

2.7. Preparation of pancreas incubation

Male Wistar rats (180–250 g) were anesthetized with sodium pentobarbital solution (50 mg/kg, i.p.) and the pancreas was isolated and perfused at 37 °C according to the method described elsewhere [18,19]. F1, F2 and purified hydroxytyrosol were dissolved in KRB buffer (pH 7.4). The pancreas were removed, transferred and then incubated in KRB buffer (pH 7.4). The perfusate was incubated for 40 min in 5 ml KRB medium at 37 °C equilibrated with 95% O2 + 5% CO2 gas. Pancreas were divided in to three sets: set 1: control, tissues incubated in KRB medium a final concentration of glucose 1 g/l; set 2: tissues incubated in KRB medium a final concentration of glucose 4 g/l; sets 3–5: slices incubated in KRB medium a final concentration of glucose 4 g/l + 50 µg/ml F1, F2 and F3, respectively. Triplicate cultures were set up for each
concentration to minimize the errors. Effluent was fractionally collected every 10 min, and its insulin concentration was measured by a radioimmunoassay kit for Bi-insulin RIA Diagnostic, Pasteur, Paris, France.

2.8. Experimental induction of diabetes

The methods of diabetes induction in rats by alloxan as described elsewhere [20]. Briefly rats were single injected intraperitoneally with a freshly prepared solution of alloxan monohydrate in normal saline at a dose (150 mg/kg BW) freshly dissolved in NaCl 0.9% buffer (pH 7). Because alloxan is capable of producing fatal hypoglycemia as a result of massive pancreatic insulin release, rats were treated with 20% glucose solution orally after 6 h. The rats were then kept for the next 24 h on 5% glucose solution bottles in their cages to prevent hypoglycemia. After 2 weeks, the rats a fatal hyperglycemia were chosen for the experiment (i.e. with blood glucose levels of 2 g/l) [20].

2.9. Experimental procedure

The rats were randomly divided into five experimental groups (n = 8). Group 1: normal rats. Group 2: diabetic rats (glycemia was superior to 2 g/l). Groups 3: diabetic rats treated with insulin 0.5 IU/rat/day (Novo Nordisk AIS, Danemak). Groups 4–6: diabetic rats received daily, respectively: OMW monomeric phenols (F1),

![HPLC chromatograms](image)

**Fig. 1.** HPLC chromatograms of OMW ethyl acetate extract (A) and the two fractions obtained from the OMW extract by means of reversed-phase SPE: F1 (B) and F2 (C). 1, hydroxytyrosol; 2, 3, unknown compounds; 4, tyrosol; 5, caffeic acid; 6, protocatechuic acid; 7, p-coumaric acid; 8, luteolin 7-O-glucosid; 9, ligstrosid; 10, luteolin; 11, elenolic acid; 12, apigenin; 13, verbascosid; 14, oleuropein; 15, rutin; P, polymeric substances.
OMW polymeric Phenols (F2) and purified hydroxytyrosol (F3) which were administrated by intraperitoneal injection i.p. at dose 20 mg/kg BW. After 2 months of treatment, the animals were sacrificed by decapitation, and the trunk blood collected. The serum was prepared by centrifugation (1500 × g, 15 min, 4 °C) and the kidneys and liver were removed, cleaned of fat and weighed; all these samples were stored at −80 °C until used. Pieces of pancreas, liver and kidney were fixed in a Bouin solution for histological studies.

2.10. Analytical methods

The lipid peroxidation in the liver and kidneys of control and all treated groups of animals was measured by the quantification of thiobarbituric acid-reactive substances (TBARS) determined by the method of Buege and Aust [21]. The activity of superoxide dismutase was assayed by the spectrophotometric method of Marklund and Marklund [22]. The GPx activity was measured by the method described by Pagila and Valentine [23]. CAT was assayed colorimet-

**Table 1**

Identification and concentrations of the principal compounds in the OMW extract fractions: F1 and F2.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Identity</th>
<th>Retention times (min)</th>
<th>λ_{max} (nm)</th>
<th>Major ESI− peaks</th>
<th>Concentration (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hydroxytyrosol</td>
<td>16.59</td>
<td>220, 277</td>
<td>153</td>
<td>1453 ± 53</td>
</tr>
<tr>
<td>3</td>
<td>Unknown</td>
<td>18.43</td>
<td>230, 286</td>
<td>193</td>
<td>n.d.</td>
</tr>
<tr>
<td>4</td>
<td>Tyrosol</td>
<td>19.34</td>
<td>219, 275</td>
<td>137</td>
<td>536 ± 43</td>
</tr>
<tr>
<td>5</td>
<td>Caffeic acid</td>
<td>26.86</td>
<td>235, 289</td>
<td>179</td>
<td>423 ± 67</td>
</tr>
<tr>
<td>6</td>
<td>p-Coumaric acid</td>
<td>29.91</td>
<td>234, 308</td>
<td>163</td>
<td>72 ± 16</td>
</tr>
<tr>
<td>8</td>
<td>PHPA^c</td>
<td>31.49</td>
<td>235, 280</td>
<td>151</td>
<td>123 ± 34</td>
</tr>
<tr>
<td>9</td>
<td>Verbasosid</td>
<td>33.42</td>
<td>216, 291, 328</td>
<td>623</td>
<td>n.d.</td>
</tr>
<tr>
<td>10</td>
<td>Luteolin</td>
<td>34.22</td>
<td>240, 265, 345</td>
<td>285</td>
<td>234 ± 56</td>
</tr>
<tr>
<td>12</td>
<td>Apigenin</td>
<td>37.86</td>
<td>235, 270, 335</td>
<td>269</td>
<td>86 ± 26</td>
</tr>
<tr>
<td>13</td>
<td>Ligstroside</td>
<td>39.12</td>
<td>217, 286, 315</td>
<td>523</td>
<td>86 ± 15</td>
</tr>
<tr>
<td>14</td>
<td>Oleuropein</td>
<td>40.18</td>
<td>228, 278</td>
<td>539</td>
<td>342 ± 22</td>
</tr>
<tr>
<td>15</td>
<td>Rutin</td>
<td>43.09</td>
<td>218, 254, 352</td>
<td>609</td>
<td>36 ± 12</td>
</tr>
</tbody>
</table>

^a Concentrations in crude OMW.
^b Not determined.
^c Para-hydroxyphenylacetic acid.

**Table 2**

Effects of OMW phenolic extracts on hepato-toxicity indices (AST, ALT, ALP, total and direct bilirubin) in plasma of diabetic rats. Values are given as mean ± S.D. for 8 rats in each group. Values differ significantly at p < 0.05.

<table>
<thead>
<tr>
<th>Groups</th>
<th>AST</th>
<th>ALT</th>
<th>ALP</th>
<th>T-bilirubin</th>
<th>d-Bilirubin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>32.7 ± 4.5</td>
<td>37.9 ± 0.7</td>
<td>157 ± 13</td>
<td>1.21 ± 0.2</td>
<td>0.16 ± 0.05</td>
</tr>
<tr>
<td>Diab + F1</td>
<td>109 ± 21^a</td>
<td>119 ± 20^a</td>
<td>243 ± 29^a</td>
<td>2.86 ± 0.3^a</td>
<td>0.66 ± 0.11^a</td>
</tr>
<tr>
<td>Diab + Ins</td>
<td>81.5 ± 15^ab</td>
<td>73.9 ± 19^ab</td>
<td>221 ± 23^ab</td>
<td>2.19 ± 0.8^ab</td>
<td>0.42 ± 0.13^ab</td>
</tr>
<tr>
<td>Diab + F2</td>
<td>73.1 ± 9^ab</td>
<td>70.5 ± 06^ab</td>
<td>195 ± 24^ab</td>
<td>1.76 ± 0.1^abc</td>
<td>0.32 ± 0.11^bc</td>
</tr>
<tr>
<td>Diab + F3</td>
<td>61.3 ± 4^abc</td>
<td>60.6 ± 07^abc</td>
<td>167 ± 10^abc</td>
<td>1.38 ± 0.1^abc</td>
<td>0.26 ± 0.03^abc</td>
</tr>
</tbody>
</table>

^a p < 0.05 as control rats.
^b p < 0.05 as diabetic rats.
^c p < 0.05 as diabetic rats treated with insulin.
^d p < 0.05 as diabetic rats treated with F1.
^e p < 0.05 as diabetic rats treated with F2.
2.11. Statistics

Data are presented as means ± S.D. The determinations were performed from 8 animals per group and the differences were examined by the one-way analysis of variance (ANOVA) followed by the Fisher test (Stat View) and the significance was accepted at p < 0.05.

3. Results

3.1. Polyphenols extraction and fractionation from OMW

OMW represents a complex medium containing mainly polyphenols of different molecular mass. Several previous investigations have demonstrated that the OMW monomeric fraction is endowed with interesting biological activities. For its recovery from OMW, we have used a liquid–liquid solvent extraction procedure. In previous investigations, it was reported that among all procedures that are employed for natural antioxidants removal, liquid–liquid solvent extraction represents a simple and convenient alternative, and it is widely used in pilot-scale production and in ultimate commercial recovery [4]. As solvent for the extraction, ethyl acetate was chosen which is frequently used to extract biophenols from aqueous matrices such as OMW. In our previous investigation, we have demonstrated that ethyl acetate exhibits a higher extraction power respect to other solvents, such as methyl isobutyl ketone, methyl ethyl ketone, diethyl ether, even though it is somewhat selective towards low (about 180 Da) and medium (about 13 kDa) molecular mass phenolic compounds [15].

The complexity of the OMW ethyl acetate extract induces researchers to make any effort in order to quantify and isolate the extracted phenolic compounds prior to the determination of their biological activities. For these purposes, fractionation of the obtained OMW phenolic extract was performed by means of a medium-pressure reversed-phase chromatographic system (see Section 2). The obtained chromatogram (data not shown) showed two separated peaks and the corresponding fractions have been collected into two different sub-fractions (F1 and F2).

3.2. Identification and quantification of phenolic compounds in the OMW fractions

The two fractions F1 and F2 resulted from the OMW extract fractionation have been characterized by HPLC analysis (Fig. 1). The obtained chromatograms (A, B, and C) showed several simple peaks corresponding to different phenolic compounds. Identification of phenolic compounds in the OMW extract fractions (F1 and F2) was preliminary performed by HPLC-UV, comparing the relative retention times and UV spectra with those of standard solutions. These standards were selected on account of their structural diversity and polarity differences and their documented presence in OMW [15,17]. The presence of formic acid in the mobile phase suppressed the dissociation of the phenolic compounds and enhanced the separation of the eluates.

### Table 3

<table>
<thead>
<tr>
<th>Groups</th>
<th>TG (g/100g)</th>
<th>T-Ch (mg/dl)</th>
<th>HDL-Ch (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.81 ± 0.02</td>
<td>2.01 ± 0.34*</td>
<td>0.53 ± 0.08</td>
</tr>
<tr>
<td>Diab</td>
<td>2.01 ± 0.34*</td>
<td>1.52 ± 0.11*</td>
<td>0.41 ± 0.03*</td>
</tr>
<tr>
<td>Diab + Ins</td>
<td>1.68 ± 0.43**</td>
<td>1.19 ± 0.14**</td>
<td>0.43**</td>
</tr>
<tr>
<td>Diab + F1</td>
<td>1.51 ± 0.05**</td>
<td>0.91 ± 0.04**</td>
<td>0.51 ± 0.03**</td>
</tr>
<tr>
<td>Diab + F2</td>
<td>1.09 ± 0.1**</td>
<td>0.86 ± 0.17**</td>
<td>0.58 ± 0.05**</td>
</tr>
<tr>
<td>Diab + F3</td>
<td>0.93 ± 0.16**</td>
<td>0.72 ± 0.19**</td>
<td>0.69 ± 0.12**</td>
</tr>
</tbody>
</table>

*abc* indicates statistical differences at *p* < 0.05.

### Table 4

<table>
<thead>
<tr>
<th>Groups</th>
<th>Creatinine (mg/dl)</th>
<th>Urea (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19.8 ± 2.1</td>
<td>0.65 ± 0.06</td>
</tr>
<tr>
<td>Diab</td>
<td>54.6 ± 8.1*</td>
<td>1.81 ± 0.24*</td>
</tr>
<tr>
<td>Diab + Ins</td>
<td>34.2 ± 6.7**</td>
<td>1.09 ± 0.19**</td>
</tr>
<tr>
<td>Diab + F1</td>
<td>33.1 ± 3.3**</td>
<td>0.75 ± 0.07**</td>
</tr>
<tr>
<td>Diab + F2</td>
<td>30.6 ± 2.3**</td>
<td>1.34 ± 0.12**</td>
</tr>
<tr>
<td>Diab + F3</td>
<td>26.5 ± 0.4**</td>
<td>0.88 ± 0.07**</td>
</tr>
</tbody>
</table>

* abcd indicates statistical differences at *p* < 0.05.
The first OMW fraction (F1) was made mainly of some monomeric biophenols: hydroxytyrosol, tyrosol, caffeic acid, protocatechuic acid and para-coumaric acid. The second OMW fraction (F2) contained principally oligomeric compounds: luteolin 7-O-glucosid, ligstrosid, luteolin, elenolic acid, apigenin, verbascosid, oleuropein, rutin and polymeric substances. Minor unidentified components were also present in F1 and F2. To our knowledge, this is the first time that verbascosid and ligstrosid were identified in the Tunisian OMW. This result shows the efficiency of the used SPE system in the separation of monomers and oligomers from the OMW extract. When there was no available standard, as with verbascosid and elenolic acid, identification was based on the comparison of their UV spectra with literature data [16,17].

Identification of phenolic compounds in the OMW extract fractions was confirmed by LC–MS, in particular for compounds which lacked of pure standards. Deprotonated molecular ions represented the base peak in the negative ion spectra of all the identified compounds as shown in Table 1. Protonated molecular ion and sodium adduct peaks were observed in the positive ion spectra of only few identified compounds, namely oleuropein and ligstrosid (data not shown). The structure assignment was based on a systematic search for molecular ions using extracted ion mass chromatograms and comparing with data in the literature [7]. For example, the elution of hydroxytyrosol at 16.59 min was confirmed by the presence of very clean and distinct peaks in both the positive and negative ion mass chromatograms at m/z 155 and 153, respectively, with sodium adduct at m/z 177 in the positive ion mass chromatogram. Furthermore, the mass spectrum of the peak at 16.59 min (Fig. 2) in the negative mode was consistent with the assignment of this peak as hydroxytyrosol by comparison with the standard mass spectrum. The presence of ion fragments at m/z 123 and 109 is due to the loss of (CH$_2$OH) and (CH$_2$–CH$_2$OH) groups, respectively. On the other hand, scanning at m/z 625 and 647 (positive ion mode) and m/z 623 (negative ion mode) produced a single peak in each of the mass chromatograms at 33.42 min. These m/z values correspond to the protonated molecular ion, sodium adduct and deprotonated molecular ion of verbascosid, respectively.

The identified compounds in the OMW extract were quantified based on standard curves, in the concentration range 50 ppm to 2000 ppm, according to the method reported by Akasbi et al. [26]. Table 1 shows the determined concentrations of biophenols. Hydroxytyrosol is the major phenolic compound in the studied OMW (1453 mg/l in crude OMW). In order to evaluate the hypoglycaemic activity of hydroxytyrosol, we were interested in the purification of this compound from the monomeric fraction (F1) using silica gel-column chromatography and prep. TLC as described in Section 2. The obtained pure hydroxytyrosol was designed as (F3).

### 3.3. Blood glucose and hepatic glycogen and OMW deriving preparations (Fig. 3)

In diabetic rats, the blood glucose level increased by 137% ($p<0.001$) compared to the control animals. In F1, F2 and F3 OMW deriving preparations treated rats, a decrease in blood glucose...
Fig. 5. Effects of monomeric and polymeric substances (F1 and F2) and purified hydroxytyrosol (F3) on renal SOD, CAT, GPX activities and TBARS of diabetic rats for 60 days. Values are given as mean ± S.D. for 8 rats in each group. Values differ significantly at \( p < 0.05 \). Statistical analysis as in legend of Table 2.

by 51, 12 and 55%, respectively after F1, F2 and F3 administration was observed. The hepatic glycogen level is lower in diabetic rats liver compared with that in normal rats, however, after F1, F2 and F3 administration, a significant increase by 67, 36 and 222%, respectively was detected compared to untreated diabetic rats.

3.4. Hepatic function, diabetes and OMW preparations

This study showed a decrease in the SOD, CAT and GPX activities in hepatic tissues of diabetic rats. In addition, diabetes reduced the HDL-Ch levels in plasma. At the same time an increase in TBARS the indices of hepatic dysfunction parameters (AST, ALT and ALP activities and direct and total bilirubin content) were obtained in plasma. The hepatic toxicity leads to problem in metabolism observed by the increase in total-cholesterol and triglycerides in plasma and liver (Tables 2 and 3).

In diabetic rats treated with F1, F2 and F3 fractions, a clear protective effect was observed in hepatic function and metabolism. In fact administration of OMW phenolic extract fractions inhibited all changes caused by alloxan-induced diabetes. This positive effect of these fractions was confirmed by histological finding (Figs. 4 and 5). As shown in Fig. 6B, fatty cysts appeared in hepatic tissues of diabetic rats indicated by arrow (Fig. 6B). However, F1, F2 and hydroxytyrosol administration to diabetic rats reduced the appearance of fat cells in liver (Fig. 6C and D). This ameliorative action is more pronounced with purified HT (Fig. 6E).

3.5. Renal function, diabetes and OMW preparations

In diabetic rats, a decrease of the SOD, CAT and GPX activities by 82, 65 and 78% in kidney at the end of the experiment was showed. Moreover, a significant increase in TBARS in kidney by 115% and creatinine and urea contents in plasma by 175 and 178%, respectively was obtained in alloxan-treated rats (Fig. 5). However, after OMW phenolic extract fractions administration, a positive action from nephropathy was clearly observed. In fact the renal SOD, CAT and GPX antioxidant activities increased after administration of OMW extract fractions. Furthermore, the OMW biophenol fractions decreased lipid peroxidation content and renal dysfunction indices such as urea and creatinine levels in plasma (Table 4). Diabetic control rat’s kidney showed fatty infiltration indicated by arrow (Fig. 7B). These changes were reduced in F1, F2 and F3 fractions treated rats (Fig. 7C, D, and E).

3.6. Pancreas, diabetes and OMW preparations

The pancreas of control rats exhibited normal islets (Fig. 8A). In alloxan-treated rats pancreas, \( \beta \) cells degeneration was observed (Fig. 8B). However, in diabetic rats treated with the monomeric biphenols (F1), the polymeric biphenols (F2) and the purified hydroxytyrosol (F3) from OMW; a patent protective action of \( \beta \) cells was recorded mainly in F3 (Fig. 8E). In vitro, Fig. 9 shows that higher glucose concentration causes a decline in insulin secretion after 40 min of pancreas incubation. In with HG concentration and F2
fraction not positive action was observed. However, in pancreas incubated at same time with HG concentration and F1 or purified hydroxytyrosol, we investigated in the first time a significant increase in the insulin secretion until the 40 Min and this positive action was more pronounced in purified HT.

4. Discussion

An accumulating body of evidence has been showing that type 1 diabetic patients manifest oxidative stress resulting from hyperglycemia, hyperinsulinemia and insulin resistance. Overwhelming free radicals generated due to oxidative stress may develop several adverse effects commonly seen in diabetes mellitus such as hepatopathy and nephropathy disorders [2,20]. As a strategy to counteract the negative effect of oxidative stress, antioxidant-based therapy is promising to minimize the complications associated with oxidative stress in diabetes mellitus. Recent observations have shown that many of these complications are diminished upon supplementation with certain dietary antioxidants such as flavonoids and polyphenols [27,28]. The present work is the first investigation dealing with the protective effects of monomeric and oligomeric biophenols as well as purified hydroxytyrosol from OMW on diabetes and their complications in liver, pancreas and kidney functions. Hydroxytyrosol, the main phenolic compound in olives and their by-products, is a phenolic compound which has been shown to possess diverse healing properties for its antioxidant and anti-inflammatory activities [29,30]. This study showed that purified hydroxytyrosol (F3) is efficient to prevent alloxan-induced
Fig. 7. (A) Normal rat kidney. (B) Diabetic treated rat kidney: tubular epithelial damage mesangial capillary proliferation and fatty infiltration. (C) Diabetic treated with insulin. (D, E and F) Diabetic rats treated with F1 or F2 or F3: (H&E 100×).

hyperglycemia. It was established that administration of hydroxytyrosol decreases the glucose level in plasma by 55% compared to untreated diabetic rats. This hypoglycaemic effect of hydroxytyrosol in diabetes could be explained by three reasons: (i) protection of pancreatic β-cells from progressive damage enhanced by alloxan and/or the enhancement of the regeneration of these cells similar to other substances such as oxovanadium [31]; (ii) like tungstate, HT can enhance insulin secretion by its insulinotropic effects; HT inhibits KATP channels and increases the voltage-dependent calcium channel which plays a key role in insulin secretion [32]; (iii) hydroxytyrosol increases peripheral uptake of glucose as was established in a previous study for oleuropein [33]; (iv) hydroxytyrosol was reported to be effective scavenger of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical as was established by Allouche et al. [29]. This antioxidant activity protects pancreatic β-cells from damage and death [34] resulting in the increase of insulin secretion which decreases glucose level in plasma (iii) HT can increase some enzymes which catalyze the phosphorylation of glucose such as hexokinase, pyruvate kinase and decrease enzymes which catalyze the dephosphorylation of glucose-6-phosphate to free glucose such as glucose-6-phosphatase and fructose-1,6-bisphosphatase [3,5,6].

The hypoglycaemic effect of hydroxytyrosol prevents glucose auto-oxidation reaction and inhibits the formation of advanced glycation endproducts (AGEs). Consequently, low rate in free radicals and more activity in antioxidant enzymes such as SOD, CAT and GPX in liver and kidney tissues were observed. The hypoglycaemic and antioxidant activities of hydroxytyrosol prevent oxidative stress
and preserve liver function as was observed by low rates in AST, ALT, ALP, total and direct bilirubin. In addition, administration of hydroxytyrosol to diabetic rats prevented kidney toxicity observed by low rates in creatinine and urea in plasma which increased in diabetic rats [38–42]. Furthermore, the protection of liver and kidney function by HT inhibited metabolic disorders in diabetic rat’s evidences by low level in Tch and TG and more HDL-Ch which linked to a lower risk of cardiovascular diseases [43]. This result is in full agreement with the findings of Gorinstein et al. [43] who reported that olive oil polyphenols decrease plasma LDL levels and prevent their oxidation in vivo. The mechanism of this hypocholesterolemic action may be due to the inhibition of dietary cholesterol absorption in the intestine or its production by liver [44] or stimulation of the biliary secretion of cholesterol and cholesterol excretion in the faeces [45]. These results agree with few human studies showing that nutritional doses of olive oil decrease TG in a group of mildly dyslipidemic patients [46,47]. In diabetic treated with the OMW monomeric fraction (F1), our study showed a good ant-diabetic and antioxidant actions. This would be related to the free radicals scavenging, anti-inflammatory and antioxidant activities of hydroxytyrosol [48–50]. These activities protect pancreatic β-cells from damage and death induced by oxidative stress as shown in Fig. 9.

The hypoglycaemic and antioxidant actions of F1 prevented the different types of oxidative damage and metabolic disorders associated with diabetes in liver and kidney.

On the other hand, our study showed that the polymeric fraction of OMW (F2) prevent diabetes and their toxicity in liver, kidney...
and pancreas. This effect principally resulted from the presence of hydroxytyrosol derivatives (oligomeric polyphenols) which are composed of hydroxytyrosol units attached to other compounds via ester or and glucosidic linkages such as verbascosidin, ligrostoside, oleuropein and salidroside [4]. In rat as in human these oligomers would be hydrolyzed by enzymes such as β-glucosidase, lipase, lipoxygenase and phenoloxidase to release hydroxytyrosol and other metabolites such as enolic acid. This statement can be supported by the findings of Fekl et al. who reported that during OMW storage the hydroxytyrosol derivatives are hydrolyzed by enzymes naturally occurring in OMW [51].

5. Conclusion

This study demonstrated the beneficial effect of hydroxytyrosol as an effective hypoglycemic and antioxidant agent in alleviating oxidative stress and free radicals as well as in enhancing enzymatic defenses in diabetic rats. The use of hydroxytyrosol may be of prophylic value in reducing the complications usually resulting from oxidative stress in diabetes mellitus.

Conflict of interest

No conflict in this work.

References

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