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HISTOPATHOLOGICAL AND OXIDATIVE FEATURES IN ALLOXAN-INDUCED DIABETIC RATS' KIDNEYS AND THE PREVENTIVE EFFECTS OF THE 3,4-DIHYDROXY-PHENYLETHANOL

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ABSTRACT

There is a big relationship between Diabete and renal dysfunctions and lesions. This study was designed to test the antidiabetic and antioxidative activities of 3,4-dihydroxy-phenylethanol in kidneys. Diabetes in *Wistar* rats was induced by intra- peritoneal injections of alloxan. The kidneys antioxidant parameters; thiobarbituric acid-reactive substances (TBARS), catalase (CAT) and *Trolox* equivalent antioxidant capacity (TEAC) were examined as well as the histological features. Diabetic rats, which have a clear hyperglycaemia, showed significant kidneys oxidative stress establishment and histological lesions. Four weeks 3,4-dihydroxy-phenylethanol administration at 8 mg/kg b.w, restored significantly the renal oxidant damages and the kidneys lesions. These results prove that the antioxidant olive leaf 3,4-dihydroxy-phenylethanol, via its important antioxidant efficiency, protect kidneys from diabetes complications which are usually associated with nephropathy or renal dysfunctions.

INTRODUCTION

Diabetes is one of the most severe problems touching health in the world. It is a disease in which the hallmark feature is elevated blood glucose concentrations due to a loss of insulin-producing pancreatic β-cells (type 1 diabetes) or through loss of insulin responsiveness in its target tissues like adipose and muscle (type 2 diabetes) (Schwarz *et al*., 2009). It affects 5% of the world population and becomes the third human killer following cancer and cardiovascular disease (Taylor, 1999). it is associated with a great number of metabolic complications and histological lesions leading with some organ failure (Remuzzi *et al*., 2006). In fact, there is a big relationship between hyperglycaemia and tissue damages, with consequent often serious diseases such as liver toxicity, lipid perturbations and renal dysfunctions (Gumieniczek *et al*., 2005; Al-Azzawie, 2006). A great number of researches focused on the implication of hyperglycaemia's oxidative stress in this large diabete related diseases (Chaudhry *et al*., 2007; Karaoz *et al*., 2002). Subsequent studies evidenced a strong link between the antidiabetic actions of some drugs with their antioxidant effects. Therefore, it has been suggested that diabete oxidative stress can be attenuated by natural components confirmed to be allowed by their important antioxidant capacity (Al-Azzawie., 2006). Nowadays, the olive tree by- products use as antioxidant source is more and more established. Furthermore, olive leaves are considered to be the source of a very efficient antioxydants such as oleuropein, 3,4-dihydroxy-phenylethanol, oleuropein aglycone and tyrosol (Jemai *et al*, 2008a, Jemai *et al*, 2008b). 3,4-dihydroxy-phenylethanol or Hydroxytyrosol is an antioxidant olive biophenol known to be with an important antioxidant, insulino-stimulatory, anti-inflammatory, hypocholesterolemic and antidiabetic effects (Hamden *et al*., 2010; Jemai *et al*., 2007). In the first part of this work we have interested with the serum and hepatic protective role of olive leaves phenolic compounds in diabetic rats, because of the important liver implication in the glucose metabolism. In this part we focused on renal affectation in relation with diabetes because of the direct implication of kidneys in glucose homeostasis.

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Moreover, there is a strong combination between diabetes and renal dysfunctions. One-third of diabetes mellitus patients develop renal alterations such as nephropathy, renal failure which are with unknown underlying mechanisms and which carries an increased mortality. Studies have identified a great number of factors that play a part in kidneys affectations in diabetes (Remuzzu *et al*., 2006). This study aims to investigate the implication of oxidative stress in the diabetic rat's kidneys lesions. The protective effects of 3,4-dihydroxy-phenylethanol rich extracts were established.

MATERIALS AND METHODS

3,4-dihydroxy-phenylethanol rich extract preparation

Olive leaves extract was prepared as previously described (Jemai *et al*., 2009). In the first step we have prepared an Oleuropein rich extract and by chemical hydrolysis we have obtained a 3,4-dihydroxy-phenylethanol rich one. The extracts were characterised by reversed-phase high-performance liquid chromatographic (HPLC).

Animals and treatments

Adult male *Wistar* rats, with body weight of 165- 195 g, were used in this study. The animals were kept in an environmentally controlled breeding room (temperature: 20 ± 2°C, humidity: 60 ± 5%, 12 h dark/light cycle). All rats had access to a standard laboratory diet (SICO, Sfax, Tunisia) and fasted overnight before blood and tissue collection. The handling of the animals was approved by the local Ethical Committee for the care and use of laboratory animals. Diabetes was induced in rats by a single intra- peritoneal injection of freshly prepared alloxan solution in normal saline at a dose of 180 mg/kg body weight (Prashant *et al*., 2005). The feeding experiment was carried out for a period of 4 weeks after the induction of diabetes in 5 days (confirmed by glucosuria). The rats were divided into 3 groups consisting of 10 ones each. Group Control (normal control) consisted of normal rats. Group Diabetic served as positive control (diabetic control) and group Hydroxy which received 8 mg/ kg of body weight of 3,4-dihydroxy-phenylethanol occurring in the hydrolyste extract. The 3,4-dihydroxy-phenylethanol rich extract was dissolved in drinking water. The duration of the treatment was 4 weeks. The body weight was measured every day. At the end of the experimental period, the rats were killed by decapitation. Organ were removed and rinsed with physiological saline solution. All samples were stored at -80 °C until analysis.

Antioxidant Enzyme Activity

Catalase (CAT) activity was evaluated in kidneys tissue. The preparation of the enzyme source fraction was as follows. One gram of kidney tissue was homogenized in 10 mL of KCl (1.15%) and centrifuged at 7740g for 15 min. The supernatants were removed and stored at -80 °C for analysis. The protein content in supernatant was measured according to the method of Bradford (Bradford *et al*., 1976) using bovine serum albumin as standard. CAT activity was measured using the method of Regoli and Principato (Regoli and Principato, 1995). Briefly, 20 µL of the supernatant was added to a cuvette containing 780 µL of a 50 M potassium phosphate buffer (pH 7.4), and then the reaction was initiated by adding 200 µL of 500 mM H_2O_2 to make a final volume of 1.0 mL at 25 °C. The decomposition rate of H_2O_2 was measured at 240 nm for 1 min on a spectrophotometer. A molar extinction coefficient of 0.0041 mM^{-1} cm^{-1} was used to determine the CAT activity. The activity was defined as the micromoles of H_2O_2 decrease per milligram of protein per minute.

Radical scavenging ability by ABTS assay

ABTS assay was performed on the kidneys homogenate supernatant. The Trolox equivalent antioxidant capacity (TEAC) assay, measuring the reduction of the ABTS radical cation by antioxidants, was derived from the method previously described (Jemai *et al*., 2009). Briefly, ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. For the study $ABTS^{\bullet+}$ solution was diluted with phosphate buffer saline pH 7.4 (PBS) to an absorbance of 0.70 (± 0.02) at 734 nm. After addition of 2 mL of diluted $ABTS^{\bullet+}$ solution to 50 µL of kidney supernatent, or Trolox standard, the reaction mixture was incubated for 6 min in a glass cuvette at 30 °C. The decrease in absorbance was recorded at 734 nm. All measurements were performed in triplicate. The free radical scavenging capacity of the biological sample, calculated as inhibition percentage of $ABTS^{\bullet+}$, was equated against a Trolox standard curve prepared with different concentrations (1.5- 30 µmol/L). The results are expressed as µM of Trolox equivalents.

Thiobarbituric Acid-Reactive Substances (TBARS) assay

TBARS are the marker of lipid peroxidation. Their concentration was measured referring to Park *et al*. (Park *et al*, 2002) assay. Briefly, 200 µL of a 10% (w/v) solution of the tissue homogenate was mixed with 600 µL of distilled H_2O and 200 µL of 8.1% (w/v) SDS, and then incubated at room temperature for 5 min. The reaction mixture was heated at 95 °C for 1 h after the addition of 1.5 mL of 20% acetic acid (pH 3.5) and 1.5 mL of 0.8% (w/v) TBA. Later, the mixture had cooled and 1.0 mL of distilled water and 5.0 mL of a butanol/pyridine (15:1) solution were added under agitation using a vortex. This solution was centrifuged at 1935g for 15 min, and the resulting coloured layer was measured at 532 nm. The concentrations were determined using a malondialdehyde (MDA) standard curve.

Histopathological analysis

At the time of sacrifice, the kidneys were removed and were fixed in a Bouin solution for 24 h and then embedded in paraffin. Sections were cut at 5 µm thicknesses and stained with haematoxylin and eosin. The sections were then viewed under photonic microscope to detect eventual histopathological changes.

Statistical analysis

Results were presented as mean ± standard deviation (S.D.). The data follow a normal distribution. A two-way analysis of

variance was performed using a Student's t-test on Microsoft Excel statistical software (Microsoft Corporation, Microsoft Office Excel 2003, Redmond, WA). The values were considered significantly different when the *p*-value was lower than 0.05.

RESULTS

Antioxidant enzymes activities

The renal antioxidant enzyme activity, catalase (CAT), significantly decreased in diabetic rats compared with the control ones (Fig. 1). The decrease was significantly restored ($p< 0.05$) by administration of 3,4-dihydroxy-phenylethanol rich extract. Hydroxytyrosol enhanced significantly this enzyme activity in diabetic animals treated for 4 weeks.

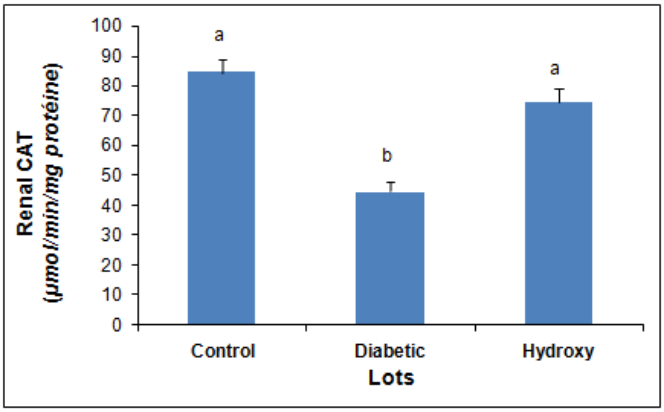

Figure 1. Effect of Hydroxytyrosol rich olive leaves extracts on the renal catalase antioxidantenzyme activity. Control: normal control; Diabetic: diabetic control; Hydroxy: Diabetic + hydroxytyrosol

ABTS assay in the kidney homogenate samples

ABTS radical cation scavenging ability (Fig. 2) in diabetic rats' kidneys was significantly low in comparison to the normal control rats. However oral administration of 3,4-dihydroxy-phenylethanol rich olive leaf extract allowed repairing the impairment between both groups. In fact there is a significant increase of the TEAC values in the rats receiving 3,4-dihydroxy-phenylethanol ($p< 0.05$).

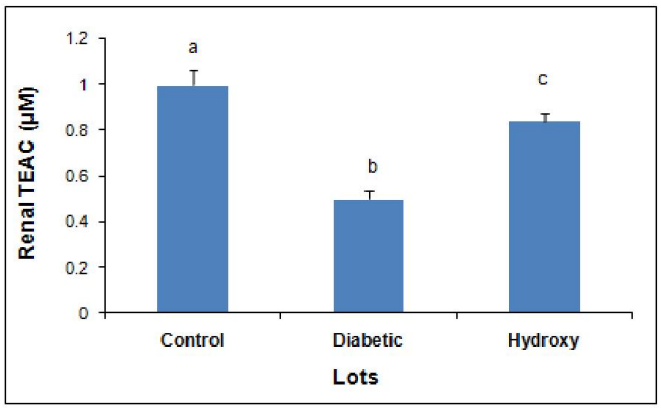

Figure 2. Effect of Hydroxytyrosol rich olive leave extract on the renal antioxidant activity (ABTS reducing activity). Control: normal control; Diabetic: diabetic control; Hydroxy: Diabetic + hydroxytyrosol

Renal lipid peroxidation

The lipid peroxidation evaluated by thiobarbituric acid reactive substances (TBARS) levels were significantly increased ($p< 0.05$) in the kidneys of diabetic rats compared to the normal control group. The administration of 3,4-dihydroxy-phenylethanol significantly reduced the TBARS concentrations ($p< 0.05$) (Fig. 3).

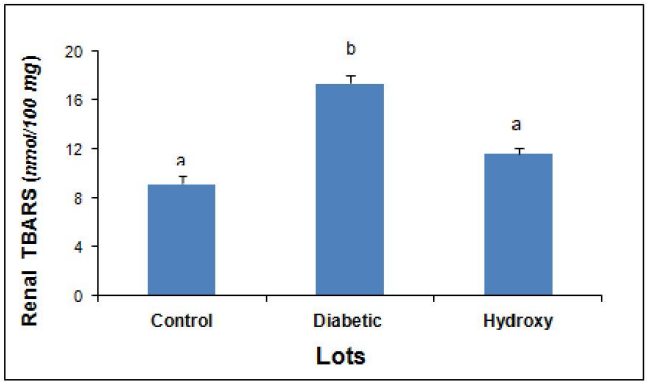

Figure 3. Effect of Hydroxytyrosol rich olive leave extract on the renal lipid peroxidation evaluated by TBARS concentrations. Control: normal control; Diabetic: diabetic control; Hydroxy: Diabetic + hydroxytyrosol

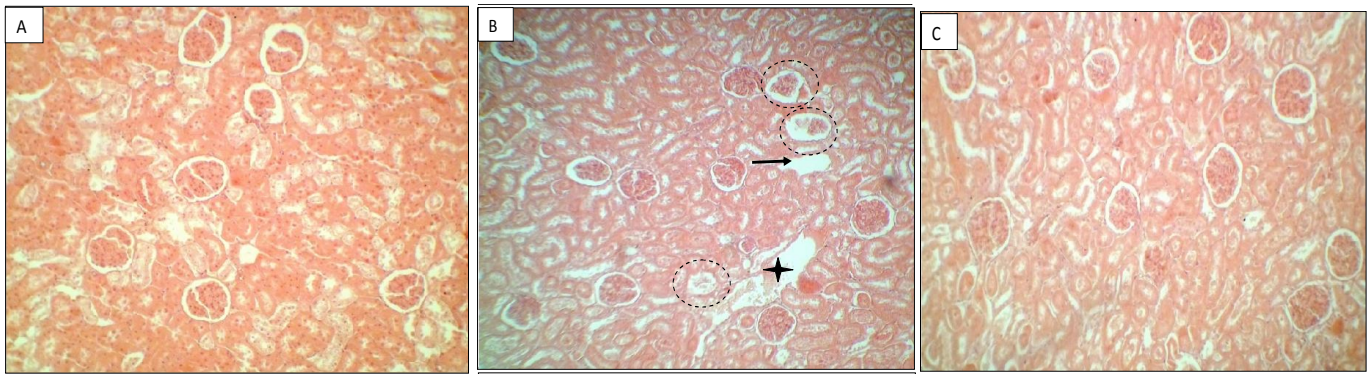

Figure 4. Kidney histological organization of Control rats (A), Diabete rats (B) and Diabete rats treated with Hydroxytyrosol (C). Fatty infiltration, dilatation of proximal tubules (arrow), degenerated glomeruli (circle) and hemorrhage (star). (H&E x100).

Histological results

In the diabetic rats kidney sections revealed tubular lesions, fatty infiltration, collapsed or occluded glomerular capillary tufts and a marked reduction in the size of the glomeruli (Fig. 4). Furthermore, hemorrhage was clearly observed. Treatment with 3,4-dihydroxy-phenylethanol rich extract markedly reduced these tubular and glomerular lesions (fig. 3). The glomeruli and the tubules were almost normal.

DISCUSSION

A great number of researches confirm the important link between oxidative stress and diabetic manifestations. In fact, free radicals are largely implicated in several different symptoms commonly seen in diabetes mellitus such as tissues damages like nephropathy disorders (Hamden *et al*., 2008; Ananthan *et al*., 2004). Subsequently, the use of natural antioxidants, such as polyphenols, as remedy in several metabolism disorders like diabetes was more and more confirmed (Kishore *et al*., 2009). In our previous researches on olive leaves extracts, we have confirmed their high content in biophenols endowed with important antioxidant activities which were efficient to restore oxidative stress lesions, hypercholesterolemia and hyperglycemia (Jemai *et al*., 2009, Bouaziz *et al*., 2005; Fki *et al*., 2007).

We have confirmed that these phenols, mainly 3,4-dihydroxy-phenylethanol, are bioactive and can be efficient in the protection against oxidative stress metabolic disorder such as diabetes. This work is the continuity of our researches on the antidiabetic effects of olive leaves biophenol in alloxan diabetic rats. It is the first investigation of the protective effects of olive leaves Hydroxytyrosol used in a very low concentration of 8mg/ Kg b.w on diabetes and their complications in kidneys. In fact Hydroxytyrosol is recently considered one of most antioxidant natural component that is why it became more and more high-priced so it is interesting to investigate its bioactive effects in lower concentration contrary to previously used in some studies (Jemai *et al*., 2009; Hamden *et al*., 2009). In this part we have interested with the protective effects of 3,4-dihydroxy-phenylethanol in the kidneys. In fact it is largely confirmed that diabetes affects kidneys in their functions as well as their histological organisations (Kumari *et al*., 1990).

Furthermore, these diabetic renal dysfunctions are highly related with oxidative stress. Our study showed that diabetic rats show a clear establishment of oxidative stress in theirs kidneys. In fact, diabetes affect significantly the different renal antioxidant parameters; In Diabetic rats, the antioxidant enzyme catalase in kidneys decreased significantly and showed very low values compared with the control rats. 3,4-dihydroxy-phenylethanol restored significantly its values and shows antioxidant protective effects in the Kidneys of diabetic rats. These results confirm the implication of oxidative stress in the diabetes. Moreover, the TEAC values as indicator of antioxidant potential presented a significant depletion in the diabetic rats kidneys compared with the normal controls. In the other hand a significant increase in lipid peroxidation expressed by TBARS was found in diabetic rats kidneys which confirmed the oxidative damages. Our results are in agreement with other findings showing that diabetes is usually combined with increase in marked oxidative impact (Duzguner *et al*., 2007). All these found perturbations in the antioxidant system were restored by the administration 3,4-dihydroxy-phenylethanol. This is with agreement with others studies which showed that biophenols increased the expression of antioxidant enzymes and subsequently enhanced the antioxidant protection parameters (Vina *et al*., 2006). Histological analysis shows that diabetes affects the organization of the kidneys which is largely confirmed previously. In fact many studies revealed the relationship between diabetes and the kidney damages (Franzen *et al*., 2014; Remuzzi *et al*., 2006). Our findings show that diabetic rats' kidneys sections revealed tubular lesions, collapsed or occluded glomerular capillary tufts as well as a marked reduction in the size of the glomeruli. Moreover, hemorrhage was clearly observed in different part of diabeticrats' kidneys sections. Treatment with 3,4-dihydroxy-phenylethanol markedly reduced these tubular and glomerular lesions. In fact, the glomeruli and the tubules were almost normal. These results are in agreement with the find of Hamden *et al* who find that biophenols can restore the kidney histology in diabetic rats (Hamden *et al*., 2009). This locally protective action of 3,4-dihydroxy-phenylethanol in the kidneys tissues can be explained by the fact that this compound has an important renal absorption in its bioavailability and it is excreted in urine as response to its orally or intravenous intake (Tuck *et al*., 2001).

Conclusion

This part of our researches confirmed the beneficial effects of 3,4-dihydroxy-phenylethanol extracted from olive tree leaves as effective antidiabetic agent protecting the kidneys from the oxidative complications and lesions related to diabetes. 3,4-dihydroxy-phenylethanol at 8 mg /kg b.w, which is a very low concentration, reduced the lipid peroxidation process, enhanced the antioxidant parameters and present a tissue protective capacity in the kidneys of diabetic rats.

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