**Oleuropein and hydroxytyrosol protect from bisphenol A effects in livers and kidneys
of lactating mothers rats and their pups'**

Asma Mahmoudi^{a#}, Hla Ghorbel^{a*#}, Zouhair bouallegui^a, Rim Marrekchi^b,
Hiroko Isoda^c, Sami Sayadi^a

a: Laboratory of Environnemental Bioprocesses, Center of Biotechnology of Sfax, University of Sfax, P.O.Box 1177, 3038 Sfax, Tunisia.

b: Laboratory of Biochemistry, CHU Habib Bourguiba Sfax 3029 Sfax, Tunisia.

c: Alliance for Research on North Africa (ARENA), Graduate School of Life and Environmental Sciences. University of Tsukuba. 1-1-1 Tennodai. Tsukuba. Ibaraki 305-8572. Japan.

*** Corresponding author:** Hla GHORBEL Ph.D.

Laboratory of Environnemental Bioprocesses,

Center of Biotechnology of Sfax,

University of Sfax,

P.O.Box 1177, 3038 Sfax, Tunisia

E-mail: ghorbelh1@yahoo.fr

: authors have equally contributed to this study

Abstract

Bisphenol A (BPA) is a chemical found in hard plastics and the coatings of food and drinks cans which can behave in a similar way to estrogen and other hormones in the human body. This study aimed to evaluate the significance of the treatment with oleuropein and hydroxytyrosol olive leaves rich extracts in reducing functional perturbations and oxidative stress arising from BPA treatment in livers and kidneys of lactating mother rats and their pups'. For this, four groups of lactating mothers were used: controls (group A), treated with bisphenol A (group B), treated with bisphenol A and oleuropein (group C) and with bisphenol A and hydroxytyrosol (group D). As results, we had found, in BPA treated group, either in mothers or in their pups', a significant decrease in morphological parameters, in catalase activity and in total antioxidant capacity associated to an increase in malondialdehyde levels in livers and kidneys. For these rats, the histological aspect showed, also, deep changes. Indeed, we had observed, in livers, hepatocellular necrosis associated to leucocytes infiltration and in kidneys tubular and glomerular necrosis. The co-treatments with BPA and oleuropein (group C) or with BPA and hydroxytyrosol (group D) ameliorate all morphological, biochemical and histological parameters as compared to BPA treated group B. The analysis of BPA and its derivatives with LC-MS/MS showed changes in their localizations between serum, livers or kidneys in all studied groups. In conclusion, the present study demonstrates the hepato-protective and reno-protective effects of oleuropein and hydroxytyrosol olive leaves extracts from BPA and its derivatives toxicity.

Key words: Bisphenol A-Bisphenol A derivatives-oleuropein-hydroxytyrosol-lactating rats-liver-kidney.

Introduction

Bisphenol A (2,2-bis-(p-hydroxyphenyl)-2-propane) (BPA), is an important industrial chemical to which humans are daily exposed and which has long been associated with endocrine disruption in experimental animal models (Li et al., 2011; Singh and Li, 2012). Several research showed that BPA was detected in the urine of adults and children (Braun et al, 2011) as well as in the serum of pregnant women (Schonfelder et al, 2002), in follicular and amniotic fluids (Ikezuki et al,2002) in cord blood and placental tissue (Schonfelder et al,2002). All these studies indicate that BPA is able to accumulate in several organs and affects many vital functions. Moreover, it has been shown that BPA was able to provoke alterations in antioxidant status and in biochemical parameters in many investigated vital organs as well as liver and kidney (Sangai and Verma, 2011; Sangai et al, 2012).

Nowadays, the use of herbal drugs, as a new strategy for alleviating the oxidative damage, is gaining popularity in the treatment of many diseases. Indeed, a growing interest has been noticed in the usage of natural antioxidants. It has been suggested that many of the negative effects of oxidative stress are diminished upon supplementation with certain dietary antioxidants such as vitamins and other non-nutrient elements as flavonoids and polyphenols (Al-Azzawie et al, 2006). Among natural antioxidants, the olive tree has been widely accepted as one of the species with the highest antioxidant activity via its oil, fruits, and leaves (Bouaziz et al, 2004). It is well known that the therapeutic activity of the olive tree is due to its richness with antioxidant phenolic components. Which are able to prevent oxidative degradations (Allouche et al, 2004). The main active components in olive leaf extracts are oleuropein and its derivative hydroxytyrosol. Several data have shown that these two compounds have various medical roles including antioxidant, anti-inflammatory and anti-apoptotic activities (Carrasco-Pancorbo et al, 2005; Fragopoulou et al, 2007).

Due to the all previously cited negative effects of BPA, it is evident that human exposure to this compound in early life, particularly during fetal and early postnatal periods is of

important concern, because of the greater vulnerability of developing organs to aberrant endocrine signal (Vandenberg et al 2009). Moreover, controlled studies of exposure effects cannot be done on pregnant women and children for ethical reasons. For these reasons it is important to utilize animal experimental studies to better understand BPA adverse health outcomes.

The aim of this study was firstly, to conduct an experimental animal model by which we have studied the toxic effects of BPA in mother rats and pups' during the lactating period and to approve the transmission of BPA and its derivatives from mothers' serum to their pups' via milk. Secondly to show that treatments with olive phenolic compounds oleuropein and hydroxytyrosol give mothers and young rats, during lactating period, hepatoprotection and renoprotection effects against BPA toxicity.

Materials and methods

2.1. Chemicals and olive leaves extracts

2.1.1. Chemicals and reagents

Bisphenol A (purity of 99.9%) was purchased from Sigma-Aldrich; ethanol was purchased from Merck (Darmstadt, Germany). Standard working solutions of BPA was prepared by dilution of the stock standard solution with ethanol.

2.1.2. Preparation of oleuropein-rich extract

The extraction was carried out on *Olea europaea* leaves, variety "Chemlali", from the south of Tunisia (Sfax prefecture). Samples of fresh green leaves were used. Leaves were dried and powdered before extraction. A mixture of methanol and water (200ml, 4:1 v/v) was added to olive leaves powder (50 g) and the mixture was kept under agitation for 24 h. Subsequently, the solution was filtered using GF/F filter paper. The extract was concentrated by evaporation to dryness at 40 °C using a rotary evaporator, and the residue obtained was stored in glass vials, at 4°C in the dark. Four grams (4 g) dry extract were dissolved in 10 ml methanol/H₂O (4:1) and extracted three times with ethyl acetate (Prolabo, France) (40 ml each time) to

prepare the oleuropein-rich extract. Ethyl acetate is then removed under vacuum. Quantitative analysis of oleuropein in the prepared extract was determined using high performance liquid chromatography (HPLC) with detection at 280 nm (**Fig 1A**).

2.1.3. Hydroxytyrosol

Hydroxytyrosol was obtained after acid hydrolysis of oleuropein rich extract. One gram of the ethyl acetate extract was dissolved in 10 ml of MeOH/H₂O (4:1) in sealed vial. The solution was hydrolyzed at 100°C for 1 h using 5ml of HCl solution (2M) (Prolabo, France). At the end of incubation, the reaction was cooled and diluted with water (10 ml) and the mixture was extracted three times with 40 ml of ethyl acetate which was subsequently removed by evaporation. Hydroxytyrosol content was determined using HPLC at 280nm (**Fig 1B**).

2.1.4. HPLC analysis

A reverse phase high performance liquid chromatography method was developed to identify and quantify the major phenolic compounds contained in the olive leaves extracts. Concentrations were calculated based on peak areas compared to those of authentic standards. The HPLC chromatograph was a Shimadzu apparatus consisting of a binary pump LC-10ATvp, anSPD-10Avp detector, a CTO-10Asvp column oven and a SIL-10Advp auto sampler. The separation was performed on a C18 column (4.6mm ID×250mm, 5 µm particle size; Shim-pack, VP-ODS). The column oven temperature was maintained at 40°C. The flow rate and the injection volume were 0.5 ml/min and 20 µl, respectively. Compounds were eluted using a gradient of phosphoric acid 0.1% in water (A) and 70% acetonitrile in water (B) for a total running time of 50 min where the gradient changed as follows: solvent B started at 10% and increased to 25% in 25 min. During the next 10 min, solvent B raised up to 80%. Then, acetonitrile reached 100% at 37 min and was maintained for 3 min. Finally, the column was reconditioned for 10 min (40–50 min) with a linear gradient of 100–10% B.

2.2. LC-MS/MS analysis of BPA and its derivatives

The detection of BPA and its derivatives was performed with the LC–MS/MS system, which consisted of a HPLC system (NANOSPACE SI-2; Shiseido, Tokyo, Japan) and a tandem mass spectrometer (TSQTM; Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization source. In fact, BPA and its derivatives were extracted using solid-phase extraction cartridge columns (OASIS HLB Extraction Cartridge 1 cm³; Waters, Milford, MA, USA), which had been washed with 15mL methanol in order to remove the BPA that originated in the columns, and subsequently extracted by liquid–liquid extraction with ethyl acetate; the organic extracts were dried under a stream of nitrogen, reconstituted in water–acetonitrile (50:50) and chromatographed using a reversed phase column (YMC-Pack Pro C18, 100mm×2mm i.d., and 3 µm in particle size; YMC Corp., Kyoto, Japan); water and acetonitrile were used as the mobile phase and the gradient elution was run at a flow rate of 0.2 ml/min; detection was performed in the negative-ion mode using selected reaction monitoring (SRM) of the m/z 227–212 transitions for BPA a collision energy of 25 eV was used for both BPA.

2.3. Animals

Rats rearing and experiments of this work were accomplished according to the European convention for the protection of vertebrate animals used for experimental and other scientific purposes (Council of Europe No. 123, Strasbourg, 1985) and according to the review committee of our institution. In the present study, adult female Swiss strain rats weighing about 200g were purchased from the Central Pharmacy (SIPHAT, Tunisia). They were housed at 22 ± 3 °C with light/dark periods of 12 h and a minimum relative humidity of 40%. They had free access to water and commercial diet (SNA, Sfax, Tunisia). After one week of acclimatization to the laboratory conditions, the female rats were caged overnight with males and the presence of spermatozoa in the vaginal smear indicated day zero of pregnancy.

2.3. Experimental protocol

The initial number of pregnant rats was 16; each pregnant rat was separated in an individual cage to deliver spontaneously three weeks after coitus. At birth, litters were reduced to eight pups each (four males and four females if possible), because it has been shown that this procedure maximizes lactation performance (Fishbeck & Rasmussen, 1987). Therefore, the total number of pups was 128. At birth day (beginning of treatments), lactating mothers and their pups were equally divided into four groups: untreated controls (group A), rats treated with BPA (250 mg/Kg) given through intramuscular injection during the treatment period (group B), rats treated with BPA and oleuropein (500mg/Kg) added in lactating mothers drinking water (group C) and rats treated with BPA and hydroxytyrosol (150mg/Kg) added in lactating mothers drinking water (group D). Sacrifice of the mothers and their pups was done at the weaning day (21st day after parturition).

2.4. Samples extraction

At the sacrifice day, for each group, all mothers (n=4) and pups (n=32) were precisely weighed. After anaesthesia with ether, livers and kidneys were carefully dissected out and weighed. Blood samples were withdrawn, in heparinised tubes, from the brachial artery of young rats and centrifuged at 2,200 ×g for 15 min. Livers, kidneys and serum samples were stored at -80°C for later biochemical and histological analysis.

2.4.1. Serum biochemical analysis

Serum samples were collected for aspartate-aminotransferase (AST), alanine-aminotransferase (ALT), lactate dehydrogenase (LDH), creatinin and urea analysis. In these samples, mineral and enzymatic analyses were done according to Roche laboratories protocols using Hitachi 912 analyzer.

2.4.2. Liver and kidney cytosol extraction

Livers and kidneys were utilized for cytosol extraction. Cells fraction was realized after adding 10 ml of KCl (1.15%) to 1g of liver or kidney tissue by using ultra-turrax at a temperature of 4°C.

2.4.3. Catalase activity of livers and kidneys

Catalase activity was assayed by the method of Claiborne (1985). Briefly, the assay mixture contained 2.4 ml of phosphate buffer (50 mM, pH 7.0), 10 µl of hydrogen peroxide (19 mM) and 50 ml enzyme source. The decrease in absorbance was measured immediately at 240 nm against reagent blank at 10 s intervals for 3 min on a Systronics Spectrophotometer. The activity of enzyme was expressed as µmol of hydrogen peroxide consumed/min per mg protein at 37 °C.

2.4.4. Total antioxidant capacity of livers and kidneys

The Trolox equivalent antioxidant capacity (TEAC) assay is measuring the reduction of the ABTS radical cation by antioxidants. ABTS radical cation (ABTS⁺) was produced by reacting 7mM ABTS stock solution with 140mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. For the study, ABTS⁺ solution was diluted with ethanol to an absorbance of 0.70 (±0.02) at 734nm. 1 ml diluted ABTS⁺ solution was added to 50 µl cytosol samples or Trolox standard. The reaction mixture was incubated for 2 min in a glass cuvette at 30°C. The decrease in absorbance was recorded at 734nm. All measurements were performed in triplicate. The free radical scavenging capacity of the biological samples, calculated as inhibition percentage of ABTS⁺, was equated against a Trolox standard curve prepared with different concentrations (40-200µmol/l). The results were expressed as mM of Trolox equivalents.

2.4.4. Determination of TBARS

As a marker of lipid peroxidation, the TBARS (thiobarbituric acid-reactive substances) concentrations were measured in kidney and liver cytosols using the method of Park et al.

(2002). For this, 200µl cytosolic sample was mixed with 600µl of distilled H₂O and 200 µl of 8.1% (w/v) SDS, vortexed, and incubated for 5 min at room temperature. The reaction mixture was heated at 95°C for 1 h after the addition of 1.5ml of 20% acetic acid (pH 3.5) and 1.5ml of 0.8% (w/v) thiobarbituric acid (TBA). After incubation the reaction was cooled and 1ml of distilled water and 5ml of butanol:pyridine (15:1) solution were added. The vortexed mixture was centrifuged at 1935×g for 15min and the resulting colored layer was measured at 532 nm using malondialdehyde (MDA) made by the hydrolysis of 1,1,3,3-tetramethoxypropane as standard.

2.4.5. LC-MS/MS analysis of BPA and its metabolites

For the LC–MS/MS analysis, three samples of serum, liver and kidney cytosols were randomly chosen from each rat groups (mothers and pups) (100 µl) were diluted with an equal volume of methanol. After centrifugation, 100 µl of acetonitrile was added to each sample, followed by a second centrifugation. From the obtained supernatant, 20 µl were taken and analyzed by LC-MS/MS.

2.4.6. Liver and kidney histopathological analysis

Three livers and kidneys were randomly selected from each group for routine light microscopy observation. They were taken and immediately fixed in a Bouin solution, embedded in paraffin and serially sectioned at 5µm. Then, the sections were stained with haematoxylin eosin (HE) for routine histological examination. In the liver, two parameters were considered for assessment, cell necrosis and portal system inflammation. In the kidney, three parameters were considered, glomerular necrosis, proximal tubular necrosis and distal tubular necrosis. Semi-quantitative assessment of liver and kidney injury was performed using scores ranging from 0 to 4 as follows: representing a 25% loss (1+), representing a 50% loss (2+), representing a 75 % loss (3+) and representing more than 75 % loss (4+) (Koop et al. 1997; Slaughter et al. 2013). The score were determined in each section selected at random and 20 fields were examined using 100X, 400X and 1000X magnification.

3. Results

3.1. Growth and feeding

3.1.1. Food and water consumption

Food and water consumptions were decreased by 27 and 36%, respectively, after treatment of mothers with BPA (group B) (**Table 1**). However, we had found a significant increase in food and water consumption by 19 and 16% for group C and by 16 and 14% for group D as compared to group B. Nevertheless, these values were still lower than the untreated control.

3.1.2. Body growth

During the studied period, a regular decrease in body growth rate was noted in BPA treated group B. So, at the sacrifice day, mothers and young rats showed a significant decrease by 11 and 40% comparatively to control group A (**Figure 2**). However, for groups C and D, we have not obtained change in body weights for lactating mothers as compared to controls. In the contrary, their pups showed body weight decrease by 28 and 31% comparatively to control rats and a partial recovery by 16 and 12% as compared to treated group B.

3.1.3. Liver and kidney weights

Following treatment of lactating mothers with BPA, while there was no significant change in liver weight recorded, a decrease in kidney weight by 15% was noticed as compared to control group A (**Table 2**). For young rats, livers and kidneys weight showed a decrease by 27% and by 42%, respectively (**Table 3**). The intakes of oleuropein (group C) or hydroxytyrosol (group D) in mothers' drinking water, in association with BPA, induced a significant increase in liver and kidney weights for mothers within groups C and D (**Table 2**). Similarly, in comparison with those of group B, a significant increase in young rats' livers by 10% and kidneys by 41% was obtained (**Table 3**).

3.2. Biochemical analysis

3.2.1. Serum biochemical parameters

Blood biochemical parameters showed important changes in mothers treated with BPA (**Table 2**, group B). Indeed, ALT, AST, creatinin, urea and LDH plasmatic levels increased by 14; 9; 29; 22 and 34% in comparison to the control group. The same trend was observed for young rats which showed an increase by 28; 12; 42; 16 and 41% for respective parameters (**Table 3**, group B). After oleuropein and hydroxytyrosol treatments (groups C and D) and for both mothers and young rats, significant correction of the adverse effects of BPA on all analyzed parameters, were noted. In fact, oleuropein reduced ALT, AST, creatinin, urea and LDH levels by 11; 18; 29; 15 and 35%, respectively, within mothers (**Table 2**, group C). Similarly, hydroxytyrosol treatment induced a respective reduction by 14; 13; 38; 25 and 37% (**Table 2**, group D). In the same way, olive bioactive molecules were able to induce, in the same trend, the correction of all parameters in young rats (**Table 3**, groups C and D).

3.2.2. Liver and kidney MDA content

The treatment with BPA caused a significant increase in MDA levels by 60 and 30% in livers, and 67 and 49% in kidneys, respectively within mothers and young rats (**Figure 3**, groups B). The consumption of olive compounds in association with BPA (groups C and D) was responsible for a statistically significant reduction of MDA contents within lactating mothers and their pups. In fact, we had obtained a total decrease of MDA contents by 30 and 50% within mothers and by 21 and 31% within young rats (**Figure 3**).

3.2.3. Catalase activity

The treatment with bisphenol A causes, in comparison with those of controls, a decrease in the catalase activity by 35; 34; 34 and 44% in livers and kidneys of mothers and young rats respectively. In groups C and D, we had obtained an improvement in catalase activity in livers and kidneys of mother and young rats by 23; 15; and 22 after oleuropein treatment and an

increase by 15; 20; 33; 17 and 30% after hydroxytyrosol treatment in comparison with those treated only with bisphenol A (**Figure 4**).

3.2.3. Liver and kidney total antioxidant capacity

In BPA treated mothers and young rats, the total antioxidant capacity showed a significant decrease by 38 and 21% in plasma and by (10; 20%) and (26; 25%) respectively in livers and kidneys as compared with those of control group A. After treatment with oleuropein and hydroxytyrosol, we had found a significant enhancement of the total antioxidant capacity in mothers and young rats. Indeed, both in mothers and their pups', after the treatment with oleuropein we had observed in serum, livers and kidneys an increase by 29; 13; 29; 24; 16 and 29% of the total antioxidant capacity. In the same way, the treatment with hydroxytyrosol induced an increase by 41; 31; 17; 24; 10 and 13 % of the TEAC (**Figure 5**).

3.2.4. BPA derivatives

The LC MS/MS analysis of serum, liver and kidney samples of BPA treated mothers and young rats showed that bisphenol A was completely absent and it was replaced by new derivatives. In fact, we had found that BPA-chlorinated derivatives were present in all tissues in BPA treated rats and were completely absent in groups C and D treated respectively by oleuropein and hydroxytyrosol (**Table 4, Table 5**). Moreover, BPA-glucuronide was found in serum and livers samples of mothers and young rats of groups B, C and D and was absent in kidneys of all treated groups (**Table 4, Table 5**).

3.2.5. Histological study

Light microscopic observation revealed that the control hepatic tissue showed normal large polygonal cells with prominent round nuclei and eosinophilic cytoplasm (**Figures 6A and 6a**). The liver histopathological observation of BPA-treated rats showed very severe hepatotoxicity as evidenced by the degeneration in hepatocytes, proliferation of Kupffer cells and a leukocytes inflammatory cells infiltration mainly in the portal triad (**Figures 6B and 6b**). Furthermore, the renal tissue showed a severe acute glomerular and tubular necrosis in

BPA-treated rats. The distal and proximal convoluted tubules are dilated with flattened epithelial lining (**Figures 7B and 8b**). The liver and kidney tissues of mother and young rats treated with oleuropein and hydroxytyrosol show an appearance similar to those of control (**Figures 6C, 6D, 7C, 7D, 8c and 8d**).

Discussion

Although a number of studies have demonstrated some toxic-effects of BPA, the present study is the first comprehensive in vivo study revealing the preventive effect of phenolic compound of olive leaves against toxicity induced by this endocrine disruptor.

Through this study, we have showed that treatment with BPA causes a significant decrease in the body weight and absolute organ weights of mothers and young rats subsequent to BPA treatment (Benjamin et al. 2003; Sangai et al. 2012). These results can be explained by the transfer of BPA and/or their derivatives through breast milk (Ye et al. 2008). Several studies have confirmed the presence of BPA in breast milk (Daniel et al 2010). Similarly, Chou and his collaborators (2011) have showed that the exposure of mothers during pregnancy at high doses of BPA causes a decrease in body weights of newborns.

For treated rats with oleuropein and hydroxytyrosol, we have found a total recovery in body growth in comparison to group B. In fact, breast milk enriched with polyphenols or carotenoids (powerful antioxidants), presents an ideal natural food for healthy growth (Li et al. 2009). In this study, phenolic compounds obtained from olive leaves confer a total body weight recovery to mothers treated with BPA and provide a good growth to their pups. Indeed, the wellbeing of young rats of groups C and D may be explained by the transfer of both phenolic compounds or/and their metabolites through breast milk.

According to our results, the treatment with BPA also causes an increase in MDA concentrations and a decrease in the CAT activity in livers and kidneys of mothers and young rats of group B. These results confirmed those of Bindhumol and his collaborators (2003) in which it was demonstrated that this endocrine disruptor leads to an increase of oxygen free

360 radical production causing lipid peroxidation. In addition, several studies showed that the
361 treatment with high doses of BPA causes a decrease in the CAT activity in the liver and
362 kidney of rats (Hassan et al. 2012; Mourad et al. 2012). However, the co-treatment with
363 oleuropein and hydroxytyrosol of BPA treated rats (groups C and D) induced a reduction in
364 MDA concentration and an improvement in catalase activity by the reduction of oxidative
365 stress. Similarly, we had found in previous results of our laboratory that the administration of
366 oleuropein and hydroxytyrosol lead to a decrease in MDA concentration and provide an
367 improvement in the activity of catalase in the liver of alloxan-diabetic rats (Jemai et al, 2009).
368 Moreover, hydroxytyrosol causes a decrease in the MDA concentration and an improvement
369 in the activity of liver and kidney catalase in cholesterol treated rats (Jemai et al, 2008).
370 Recently, Alirezai and his collaborators (2012) have showed that oleuropein provides a
371 reduction in the MDA concentration and an improvement in the activity of liver catalase in
372 ethanol treated-rats. Furthermore, Tavafi et al (2012) have demonstrated that the treatment
373 with oleuropein induces a decrease in the MDA concentration and an increase in the catalase
374 activity in the kidney of gentamicin treated rats.

375 The histological study shows that the BPA treatment induced severe hepatotoxicity as
376 evidenced by hepatocytes necrosis with leucocytes inflammatory cells infiltration mainly in
377 the portal triad. The important occurrence of hepatic lesions in this area could be explained by
378 the accumulation of high levels of BPA near the portal triad system. Moreover, all of these
379 changes have been revealed by the increase in ALT, AST and LDH levels of BPA treated rats.
380 Indeed, Hassan et al (2012) showed that the treatment of rats with increasing doses of BPA
381 causes, in dose dependent manner, an increase in transaminases plasma levels. This increase
382 in plasma transaminase levels may be related to the hepatotoxicity caused by the induction of
383 oxidative stress (Mourad et al, 2012). In addition, Korkmaz et al (2010) have showed that the
384 treatment of rats with BPA causes an increase in LDH plasma levels. For rats of groups C and
385 D, the treatment with the phenolic compounds of olive leaves induced a significant correction

of AST, ALT and LDH levels. These data confirmed that oleuropein and hydroxytyrosol prevent the adverse effects of BPA through the modulation of transaminases levels and protect tissues against BPA induced injuries as evaluated by LDH concentrations (Domitrović et al. 2012; Alirezai et al. 2012)

The renal tissue showed in BPA treated group a severe glomerular and tubular necrosis both in proximal and distal parts. The distal and proximal convoluted tubules are dilated with flattened epithelial lining. These morphological changes were confirmed by the increase in the plasma concentrations of urea and creatinine following BPA treatment. These results are in agreement with those performed by Singh et al (2011) who showed that treatment of rats with bisphenol A diglycidyl ether causes an increase in plasma levels of creatinine and urea which is associated to renal dysfunction. More interestingly, we have found that supplementation with phenolic compounds of olive leaves of BPA-treated rat's causes a remediation in creatinine and urea plasma levels. These results are consistent with those of Tavafi and his colleagues (2012) who have showed that the administration of oleuropein in gentamicin treated rats led to an improvement in renal function and a decrease in the creatinine and urea plasma levels. Similarly, the treatment with hydroxytyrosol of diabetic rats protects against renal dysfunction and provides attenuation in creatinine and urea plasma levels (Hamden et al, 2009).

As is well known, BPA is detoxified by the generally recognized metabolic pathways at the level of the liver by providing a molecule with a hydrophilic character. This process will facilitates its solubilization and elimination by the renal excretion way. In mammals, many metabolic pathways lead to BPA-glucuronide generation (Migeot et al, 2013). In this study, we have detected by LC MS/MS the presence of BPA glucuronide in the serum and livers of all treated groups rats but not in the kidneys, which confirms their hydrophilic character and make easier their excretion in urine. Moreover, there where another detoxification pathway that leads to Clx-BPA formation which is more lipophilic than the glucorinated form (Migeot

et al, 2013). In fact, we had found in mothers and young rats treated with BPA the presence of mono-, di- and tri-chloro-BPA in the serum, liver and kidney samples. This proves that chlorinated derivatives of BPA accumulate in tissues (Fernandez et al, 2007). In fact, our results are in agreement with those of Liao and his collaborators (2012) who reported a poor excretion of Clx-BPA by the urine and their accumulation in several tissues. All perturbations obtained in lactating young rats could be explained by the transmission of Clx-BPA from mothers blood to their pup's via milk. Indeed, because of its fat composition, breast milk is known as a relevant uptake route for lipophilic compounds as Clx-BPA (Migeot et al, 2013). As a result, the accumulation of Clx-BPA, which is more toxic than BPA, in mothers and young rats tissues leads to an increase in the oxidative stress and plays a central role in acute liver and kidney damages as it was demonstrated by our results (Reyes-Gordillo et al 2007; khan et al 2011).

However, for groups C and D, the co-treatment by BPA and phenolic compounds of olive leaves induced a total disappearance of chlorinated BPA from the serum, liver and kidney samples. In fact, the disappearance of these chlorinated derivatives could be explained by the general ameliorative effects of oleuropein and hydroxytyrosol on the renal excretion (Deiana et al, 2008).

In conclusion, polyphenols olive leaves rich extract, is able to protect against the toxicity of BPA, not only by enhancing the antioxidant system activity in cells, but also by blocking the formation and/or increasing the urinary excretion of the toxic derivatives of BPA.

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Figures legends:

Figure 1: HPLC chromatograms at 280 nm of olive leaf extracts (A) olive leaf extract after acid hydrolysis (B). (1): Oleuropein; (2) hydroxytyrosol.

Figure 2: Body weight of mothers and young rats: controls (group A), treated with bisphenol A (group B), bisphenol A and oleuropein (group C) and bisphenol A and hydroxytyrosol (group D).

Treated (B, C, D) vs. control (A): ***: $p \leq 0.001$, **: $p \leq 0.01$, *: $p \leq 0.05$

Treated (C, D) vs. treated (B): +++: $p \leq 0.001$, ++: $p \leq 0.01$, +: $p \leq 0.05$.

Figure 3: MDA levels (nmole/100 g) in liver and kidney in mothers and young rats, control (group A), treated with bisphenol A (group B), bisphenol A and oleuropein (group B), bisphenol A and hydroxytyrosol (D).

Treated (B, C, D) vs. control (A): ***: $p \leq 0.001$, **: $p \leq 0.01$, *: $p \leq 0.05$

Treated (C, D) vs. treated (B): +++: $p \leq 0.001$, ++: $p \leq 0.01$, +: $p \leq 0.05$

Figure 4: Catalase activity in livers and kidneys of mother and young rats, control (group A), treated with bisphenol A (group B), bisphenol A and oleuropein (group B), bisphenol A and hydroxytyrosol (D).

Treated (B, C, D) vs. control (A): ***: $p \leq 0.001$, **: $p \leq 0.01$, *: $p \leq 0.05$

Treated (C, D) vs. treated (B): +++: $p \leq 0.001$, ++: $p \leq 0.01$, +: $p \leq 0.05$

Figure 5: Trolox equivalent antioxidant capacity (nmole/100 g tissue) in plasma, livers and kidneys in mothers and young rats, control (group A), treated with bisphenol A (group B), bisphenol A and oleuropein (group B), bisphenol A and hydroxytyrosol (D).

Treated (B, C, D) vs. control (A): ***: $p \leq 0.001$, **: $p \leq 0.01$, *: $p \leq 0.05$

Treated (C, D) vs. treated (B): +++: $p \leq 0.001$, ++: $p \leq 0.01$, +: $p \leq 0.05$

Figure 6: Histological aspect and semi-quantitative scores of liver injuries (6e) of lactating mothers and young rat's livers in control group (A, a), treated with bisphenol A group (B, b), treated with bisphenol A and oleuropein group (C, c) and with bisphenol A and hydroxytyrosol group (D,d).

HE staining; magnification: 400X, 1000X; HN: Hepatocytes necrosis; KC: Kupffer cells

Treated (B, C, D) vs. control (A): ***: $p \leq 0.001$, **: $p \leq 0.01$, *: $p \leq 0.05$

Treated (C, D) vs. treated (B): +++: $p \leq 0.001$, ++: $p \leq 0.01$, +: $p \leq 0.05$

Figure 7: Histological aspect and semi-quantitative scores of kidney injury (7e) of lactating mothers kidney in control group (A), treated with bisphenol A group (B), treated with bisphenol A and oleuropein group (C) and with bisphenol A and hydroxytyrosol group (D).

HE staining; magnification: 100X 400X, 1000X; GN: glomerular necrosis; PN: proximal tubular necrosis; DN: distal tubular necrosis

Treated (B, C, D) vs. control (A): ***: $p \leq 0.001$, **: $p \leq 0.01$, *: $p \leq 0.05$

Treated (C, D) vs. treated (B): +++: $p \leq 0.001$, ++: $p \leq 0.01$, +: $p \leq 0.05$

Figure 8: Histological aspect and semi-quantitative scores of kidney injury (8e) of lactating young rats livers in control group (a), treated with bisphenol A group (b), treated with bisphenol A and oleuropein group (c) and with bisphenol A and hydroxytyrosol group (d).

HE staining; magnification: 100X, 400X, 1000X; GN: glomerular necrosis; PN: proximal tubular necrosis; DN: distal tubular necrosis

Treated (B, C, D) vs. control (A): ***: $p \leq 0.001$, **: $p \leq 0.01$, *: $p \leq 0.05$

Treated (C, D) vs. treated (B): +++: $p \leq 0.001$, ++: $p \leq 0.01$, +: $p \leq 0.05$

Table 1: Daily food, water, oleuropein and hydroxytyrosol intake by lactating mothers rats. controls (group A), bisphenol A treatment (group B), bisphenol A and oleuropein (group C), and bisphenol A and hydroxytyrosol (group D).

Parameters and treatments	A	B	C	D
Food consumption (g/day/mother)	41±2	30±3 ^{***}	37±3 ^{***+++}	36±3 ^{***+++}
Water consumption (ml/day/mother)	56±5	36±4 ^{***}	43±2 ^{***+++}	42±3 ^{***+++}
Ingested oleuropein (mg/day/mother)	-	-	4.35±0.41	-
Ingested hydroxytyrosol (mg/day/mother)	-	-	-	0.84±0.73

Number of determinations (n = 22); *** P ≤ 0.001: groups B, C and D versus control group A; +++ P ≤ 0.001: groups C and D versus group B.

Table 2: Liver and kidney weights (g), serum ALT (UI/L), AST (UI/L), creatinine (μmol/l), urea (mmol/l) and LDH (UI/L) levels in lactating mothers rats: controls (group A), bisphenol A treatment (group B), bisphenol A and oleuropein (group C), and bisphenol A and hydroxytyrosol (group D).

Parameters and treatments	A	B	C	D
Liver weights (4)	10.02 ± 0.99	9.20±0.95	10.34±1.20	9.63±0.89
Kidney weight (4)	0.8±0.008	0.68±0.028***	0.78±0.017+++	0.79±0.011+++
ALT(4)	31.5±1.5	36.5 ± 0.5*	32.5 ± 0.5 ⁺	31.5±0.5 ⁺
AST(4)	126.75±4.26	139.5±3.5	113.75±6.29	121±5
Creatinine (4)	28.56±2	40.46±4.7***	28.71±3.03+++	24.98±0.11+++
Urea (4)	10.05±0.45	12.76±0.09***	10.86±0.32+++	9.66±0.62+++
LDH (4)	515.2 ±21.21	773.5±28.99**	505.1±35.35 ⁺⁺	483.5±11.5 ⁺⁺

Number of determinations (n = 22)

*** P ≤ 0.001: groups B, C and D versus control group A

+++ P ≤ 0.001: groups C and D versus group B.

Table 3: Liver and kidney weights (g), serum ALT (UI/L), AST (UI/L), creatinine (μmol/l), urea (mmol/l) and LDH (UI/L) levels of 21days old young rats: controls (group A), treated with bisphenol A (group B), bisphenol A and oleuropein (group C), and bisphenol A and hydroxytyrosol (group D)

Parameters and treatments	A	B	C	D
Liver weights (14)	1.55±0.08	0.60±0.08***	1.01±0.04*** ⁺⁺	1.02±0.10*** ⁺⁺
Kidney weight (20)	0.19±0.003	0.11±0.011***	0.14±0.009*** ⁺⁺	0.18±0.015*** ⁺⁺
ALT (6)	21 ± 1	29 ± 2**	20 ± 1 ⁺⁺	22 ± 2 ⁺⁺
AST (6)	126.5±0.5	143±1**	128.66±3.68 ⁺⁺	127±1.41 ⁺⁺⁺
Creatinine (6)	24.36±2.34	41.82±1.15***	35.61±0.74 ⁺⁺	34.79±0.82 ⁺⁺
Urea (6)	6.5±0.17	7.73±0.12***	6.85±0.17 ⁺⁺⁺	6.8±0.12 ⁺⁺⁺
LDH (6)	372±31.11	632.33±43***	442±38.60 ⁺⁺⁺	543.5±2.12 ⁺⁺⁺

Number of determinations ()

Treated B, C and D versus controls A: * $P \leq 0.05$ ** $P \leq 0.01$ *** $P \leq 0.001$

Treated C and D versus treated B: ⁺ $P \leq 0.05$ ⁺⁺ $P \leq 0.01$ ⁺⁺⁺ $P \leq 0.001$

Table 4: MS/MS determination of BPA and BPA derivatives in serum, liver and kidney samples of mother and young rats of control group A, bisphenol A treated group B, bisphenol A and oleuropein treated group C and bisphenol A and hydroxytyrosol treated group D.

Derivatives	Molecular weight	Fragments
BPA	227.0–212.0	_CH3
	227.0–133.0	_H, _C6H5O
BPA-Gluc	403.2–227.0	_C6H8O6
	403.2–113.0	_H, _H2O, _CO2, _C15H15O2
BPA-Cl	263-227	_ Cl
BPA-Cl2	297.0-279.0	_ H2O
BPA-Cl3	331.0-279.0	_H2O, _ Cl

Table 5: BPA and BPA derivatives repartition in serum, liver and kidney samples of mother and young rats of different treatments: controls (group A), bisphenol A treatment (group B), bisphenol A and oleuropein (group C), and bisphenol A and hydroxytyrosol (group D).

Mother and young rats		BPA	BPA-Gluc	BPA-Cl	BPA-CL2	BPA-Cl 3
Serum	Group B	-	+	+	+	+
	Group C	-	+	-	-	-
	Group D	-	+	-	-	-
Livers	Group B	-	+	+	+	+
	Group C	-	+	-	-	-
	Group D	-	+	-	-	-
Kidneys	Group B	-	-	+	+	+
	Group C	-	-	-	-	-
	Group D	-	-	-	-	-

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718 **Figure 1:**

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Figure 2

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809 **Figure 7 :**