

Papaya fruits extracts enhance the antioxidant capacity and modulate the genotoxicity and oxidative stress in the kidney of rats fed ochratoxin A-contaminated diet

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ABSTRACT

Ochratoxin A (OTA) is a mycotoxin widespread contaminates food and has potent nephrotoxicity. The present study aimed to determine total phenolics and DPPH radical scavenging activity for water and ethanolic extracts (WE and EE) of papaya fruits and evaluation of the protective role of these extracts against OTA-induced oxidative damage and nephrotoxicity in rats. Sixty male Sprague-Dawley rats were divided into six groups and treated for 21 days as follow: the control group, OTA-treated group (3 mg/kg diet), WE or EE extracts treated groups (250 mg/kg b.w) and OTA plus WE or EE-treated groups. Blood and kidney samples were collected for different biochemical, cytogenetical and histological analyses. The results showed that WE and EE contained 408.54 and 296.85 g/kg total phenolic, respectively and DPPH radical scavenging activity was higher in WE than EE. Animals fed OTA-contaminated diet showed signs of toxicity as indicated by the significant decrease in body weight gain and food intake, the disturbances in kidney biochemical indices, increase DNA fragmentation and oxidative stress markers, the decrease in antioxidant enzymes activities and gene expression as well as severe histological and histochemical changes in the kidney tissues. Both extracts succeeded to counteract these alterations and WE was more effective than EE. These results concluded that papaya fruits extracts succeeded to a great extent to counteract the oxidative stress of OTA and to protect the kidney against its toxic effects and may be promising candidate as food supplement for the protection against OTA in high endemic area.

INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin produced by *Aspergillus ochraceus* in the warm and the tropical countries and by *Penicillium verrucosum* in temperate climates (Santos *et al.*, 2010; Iqbal *et al.*, 2013). OTA has classified by IARC as group 2B carcinogen, probable human carcinogen (IARC, 2002) and can contaminate several agriculture crops, especially grains. This mycotoxin is stable to heat treatment and can enter the food chain either through the raw or the processed products in addition to

through the animal-origin products derived from the livestock fed with contaminated feed (EFSA, 2006; FAO, 2012). OTA exposure is a worldwide problem and it is detected in human sera in several countries (Duarte *et al.*, 2011; Märtlbauer *et al.*, 2009; Soto *et al.*, 2016). Previous studies have indicated that exposure to OTA results in a number of diseases in both animals and humans and mainly affect the kidney. Moreover, it is also responsible for neurotoxicity, immunotoxicity, myelotoxicity, teratogenicity and reproductive toxicity in different animal species (Heussner and Bingle, 2015; Cariddi *et al.*, 2016; Abdel-Wahhab *et al.*, 2016; Costa *et al.*, 2016). OTA is also responsible to Balkan endemic nephropathy (BEN) and associated with urothelial tumors in human (Pfohl-Leszkowicz *et al.*, 2007).

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The World Health Organization (WHO, 2008) suggested five hypotheses as main contributors (collectively or individually) to the mode of action of OTA including (1) the genotoxicity resulted from direct interaction of OTA or its reactive metabolite with DNA; (2) generation of tumors secondary to chronic renal toxicity and compensatory cell proliferation; (3) generation of tumors secondary to inhibition of phenylalanine-tRNA^{Phe}-synthetase and protein synthesis; (4) mitochondrial dysfunction leading to oxidative stress and indirect induction of DNA damage; and (5) disruption of cell-cell signaling pathways and the process of cell division. The EFSA (European Food Safety Authority) and the JECFA (Joint FAO/WHO Expert Committee on Food Additives) have set a Tolerable Weekly Intake (TWI) and Provisional Tolerable Weekly Intake (PTWI) for OTA at 120 (EFSA, 2006) and 100 ng/kg body weight (JECFA, 2008), respectively. Papaya (*Carica papaya* L.) is a fruit cultivated widely all over the world, particularly in tropical countries. It is extremely appreciated because of its sensory characteristics, its highly nutritional value and digestive characteristic (Paes *et al.*, 2015). Papaya fruits contain two main biologically active compounds namely papain and chymopapain which are widely used for the treatment of digestive disturbance (Huet *et al.*, 2006). These fruits also contain caricain, and glycerin endopeptidase which have the ability to improve the acidic pH conditions and the degradation of pepsin (Kokila *et al.* 2016).

Moreover, papaya contains other active compounds including a variety of phytochemicals such as natural phenols (Khan *et al.*, 2012) and polyphenols which inhibit the oxidation of biomolecules through their free radicals scavenging properties (Urquiaga and Leighton, 2000) or through the enhancement of endogenous antioxidant enzymes activities (La Marca *et al.*, 2012) consequently, prevent the onset of different chronic diseases in humans. According to Dominguez de Maria *et al.* (2006), papaya fruits are rich in lipase and ahydrolase which are tightly bonded to the water-insoluble fraction of crude papain thus consider as a “naturally immobilized” biocatalyst. The aims of the present study were to determine total phenolics and DPPH radical scavenging activity for water and ethanolic extracts of papaya fruits and evaluation their protective effects against oxidative stress and renal toxicity in rats fed OTA-contaminated diet.

MATERIALS AND METHODS

Chemicals and kits

OTA standards and 1,1-Diphenyl-2-picrylhydrazyl (DPPH), sodium tripolyphosphate (TPP) and RevertAidTM H Minus First Strand cDNA Synthesis Kit were purchased from Sigma Chemical Co. (St. Luis, Mo, USA). Kits of creatinine and urea were obtained from Randox Laboratories LTD Co. (UK.). Total antioxidant capacity (TAC) and lipid peroxidation (MDA) kits were obtained from Biodiagnostic Co. (Giza, Egypt). TRIZOL reagent was purchased from InvitrogenTM (Carlsbad, CA, USA). All other chemicals were of the highest purity commercially available.

Plant materials

Papaya (*Carica papaya* L.) fruits were collected from a private plantation located in Qalubia region (Egypt) during June 2015. The fruits were identified in the Fruits Department, National Research Centre and the voucher was kept in the herbarium of NRC. The amount of plant used was 500 g.

Preparation of papaya extracts

Fresh fruits of papaya were cut into small pieces, dried and finally ground with a blender into powder form. A crude ethanolic extract was prepared by soaking and stirring the powder in absolute ethanol (200 g/500 ml ethanol) for 3 days. The extract was filtered and the residue was re-extracted twice using ethanol. The pooled extract was vacuum-dried at 40 °C. Aqueous extract was prepared by maceration process. The filtrate was subjected to lypholization process using Freeze Dryer system (Dura-Dry Freeze Dryer, Model PAC-TC-V4; FTS system, Inc., Stone Ridge, NY, USA) under pressure, 0.1 to 0.5 mbar and temperature -35 to -41°C conditions. Both the dry ethanolic and aqueous extracts were stored at -20 °C until analysis. These procedures resulted in an approximate yield of 13% (w/w) of ethanolic extract (EE) and 27 % (w/w) of aqueous extract (AE) based on the dry weight.

Determination of total phenolic compounds

Five mg of the extract were dissolved in a 10 ml mixture of acetone and water (6:4 v/v). Samples (0.2 ml) were mixed with 1.0 ml of 10- fold diluted Folin-Ciocalteu reagent and 0.8 ml of sodium carbonate solution (7.5%). After 30 min at room temperature, the absorbance was measured at 765 nm using V-530 UV/visible spectrophotometer. Estimation of phenolic compounds as catechin equivalents was carried out using standard curve of catechin (Jayaprakasha *et al.*, 2003).

Determination of radical scavenging activity by DPPH assay

Certain of crude extracts were dissolved in methanol to obtain a concentration of 200 ppm. A volume of 0.2 ml of this solution was completed to 4 ml by methanol and 1 ml DPPH solution (6.09×10^{-5} mol/L) in the same solvent was then added. The absorbance of the mixture was measured at 516 nm after 10 min standing. The reference sample (blank) was 1 ml of DPPH solution and 4 ml methanol. Triplicate measurements were made and the antioxidant activity was calculated by the percentage of DPPH that was scavenged according to Nogala-Kalucka *et al.* (2005).

Ochratoxin A (OTA) production

OTA was produced through the fermentation of corn by *Aspergillus ochraceus* (NRRL 3174) as described by Varga *et al.* (1996). The fermented corn was autoclaved, ground to a powder and the OTA content was measured by the high-performance liquid chromatography (HPLC) according to Xiao *et al.* (1995). The corn powder was incorporated into the basal diet to provide the desired level of 3 mg OTA/kg diet. The diet containing OTA was analyzed and the presence of OTA was confirmed by HPLC.

Experimental animals

Three months old male Sprague-Dawley rats (120-130 g) were purchased from the Animal House Colony, Giza, Egypt) and were maintained on standard lab diet (protein: 160.4; fat: 36.3; fiber: 41 g/kg and metabolizable energy 12.08 MJ) in artificial illuminated and temperature controlled room free from any other source of chemical contamination at the Animal House Laboratory, National Research Center, Dokki, Cairo, Egypt. After an acclimatization period of 1 week, the animals were divided into six groups (10 rats/group) and housed in filter-top polycarbonate cages. All animals were received humane care in compliance with the guidelines of the Animal Care and Use Committee of the National Research Center, Dokki, Cairo, Egypt and the National Academy of Sciences (NIH publication 86-23 revised 1985).

Experimental design

Animals within treatment groups were maintained on their respective diets for 3 weeks as follows: group 1, untreated control; group 2, fed OTA-contaminated diet (3 mg/kg diet); group 3, treated orally with EE (250 mg/kg b. w.); group 4, treated orally with AE (250 mg/kg b.w.); groups 5 and 6, fed OTA-contaminated diet and treated orally with EE or AE. The animals were fed restrictively (~0.8 of *ad libitum* intake, 20 g/d) to avoid differences in OTA intake and were observed daily for any signs of toxicity during the experimental period. Food intake was recorded each other day and body weight gain was recorded twice a week throughout the experimental period. At the end of the experimental period, fasting blood samples were collected from the retro-orbital venous plexus under ether anesthesia. The blood samples were left to clot and then centrifuged at 3000 rpm for 15 minutes to separate sera which were used for the determination of different biochemical parameters according to the kits instructions using spectrophotometer. After the collection of blood samples, all animals were rapidly killed and a whole kidney of each animal was dissected and divided longitudinal into two halves. The first half was used for the molecular analyses and the second half was washed with saline and immediately homogenized in ice-cold buffer containing 50 mM tris-HCl and 300 mM sucrose (pH 7.4) to give 10% w/v homogenate (Tsakiris *et al.*, 2004). The homogenate was centrifuged at 3000 rpm at 0°C for 10 minutes and the supernatant was stored at -20 °C to the second day until analysis. This supernatant (10%) was used for the determination of malondialdehyde (MDA) by the spectrophotometric method described by Ruiz-Larrea *et al.* (1994), total antioxidant capacity (TAC) levels as described by Koracevic *et al.* (2001) and Na⁺/K⁺-ATPase activity according to the modified chemical method that described by Tsakiris *et al.* (2000). The homogenate was further diluted with phosphate buffer solution to give 2% and 0.5% dilutions for the determination of renal GPx (2%) and SOD (0.5%) activities. The second whole kidney from each animal was fixed in 10% neutral formalin and paraffin embedded. Sections (5 µm thickness) were stained with hematoxylin and eosin (H & E) for the histological examination. Other sections from kidney were stained with Bromophenol blue for the determination of total

protein content in the whole kidney tissue (Drury and Wallington 1980).

Molecular analyses

DNA fragmentation

Samples of the second half of each kidney were lysed in 0.5 ml of lysis buffer containing 10 mM tris-HCl (pH 8), 1 mM EDTA and 0.2 % triton X-100. The content was centrifuged at 10,000 rpm for 15 min at 4 °C to separate intact chromatin in the pellet from fragmented/damaged DNA in the supernatant. The pellets were resuspended in 0.5N perchloric acid (P) and 5.5N perchloric acid was added to the supernatants (S) to obtain a final concentration of 6.0 N. Samples were incubated at 90 °C for 20 min and centrifuged at 10,000 rpm for 10 min to remove proteins. A volume of 160 ml of diphenylamine (DPA) solution [150 mg DPA in 10 ml glacial acetic acid, 150 ml of sulfuric acid and 50 ml acetaldehyde (16 mg/ ml)] was added to each sample and incubated at room temperature for 24 h (Gibb *et al.*, 1997). The absorbance was measured at 600 nm using a UV doublebeam spectrophotometer (Shimazu Co. Ltd., Tokyo, Japan). The proportion of fragmented DNA was calculated from absorbance reading at 600 nm using the formula:

$$\% \text{ Fragmented DNA} = [\text{OD(S)} / \text{OD(S)} + \text{OD(P)}] \times 100$$

Where: OD(S) optical density of supernatant

OD(P) optical density of pellet

RNA extraction

Samples of frozen kidney tissues (50-100 µg) were thawed and homogenized in TRIZOL reagent. The solution of extracted RNA was recovered in 100 µl molecular biology grade water. The total RNA samples were pretreated by DNA-free™ DNase to remove any possible genomic DNA contamination according to manufacturer's protocol. The quality of RNA was determined using spectrophotometer at 260 nm by UV visualization of an ethidium bromide-stained agarose-formalin gel.

Reverse transcription

Single strand cDNA was synthesized for PCR (Spectrum Laboratory Products, Inc. (New Brunswick, NJ, USA) and semi-nested PCR purposes using total RNA isolated from kidney tissues and oligo(dT)₁₈ as primer for reverse transcriptase. The reaction mixture of 20 µl used in the 1st step of cDNA synthesis included 4 µl of total RNA (2 µg), 0.5 µl oligo(dT)₁₈ (10 µM), and 5 µl of DEPC-treated water. The reaction mixture was incubated at 70 °C for 5 min. A 4 µl of 5X M-MuLV-Reverse Transcriptase buffers, 2 µl of 10 mM dNTP mix, 0.5 µl of dH₂O and 0.5 µl (20 units) of ribonuclease inhibitor was added to the above reaction mixture. The above mixtures were further incubated at 37 °C for 5 min and 2 µl M-MuLV-Reverse Transcriptase (40 units) was then added and cDNA was synthesized for 60 min at 42 °C. The reaction was stopped by heating the reaction mixture at 70 °C for 10 min. The synthesized cDNA was stored at -20°C.

Table 1: Details giving primer sequences and expected product sizes for the genes amplified.

cDNA	Genbank Accession No.	Forward primer	Reverse primer	RT-PCR product size
β -actin	V01217	5'-CTGCTTGCTGATCCACA	5'-CTGACCGAGCGTGGCTAC	505bp
Cu-Zn SOD	X05634	5'-GCAGAAAGCAAGCGGTGAAC	5'-TAGCAGGACAGCAGATGAGT	387bp
GPx I	M21210	5'-CTCTCCGCGGTGGCACAGT	5'-CCACCACCGGGTCGGACATAC	290bp

Cu-Zn SOD – copper zinc superoxide dismutase; GPx I- glutathione peroxidase

Polymerase chain reaction (PCR)

The first-strand cDNA from different kidney samples was used as the template for amplification by the PCR with the pairs of specific primers presented in Table (1) (from 5'to3') according to Limaye *et al.* (2003). β -actin, a house-keeping gene was used for normalizing mRNA levels of the target genes. The PCR cycling parameters were one cycle of 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 70 °C for 40 s, and 72 °C for 5 min for Cu-Zn SOD using hydrogen peroxidase and for GPx I gene using horseradish peroxidase substrate according to the instructions supplied by the producer company. The PCR product was run on a 1.5 % agarose gel in Tris-borate-EDTA buffer and visualized over a UV Trans-illuminator. The ethidium bromide-stained gel bands were scanned and the signal intensities were quantified by the computerized Gel-Pro (version 3.1 for window 3). The ratio between the levels of the target gene amplification product and the β -actin (internal control) was calculated to normalize for initial variation in the sample concentration as a control for reaction efficiency (Raben *et al.*, 1996). All PCRs were independently replicated three times.

Statistical analysis

All data were statistically analyzed using the General Linear Models Procedure of the Statistical Analysis System (SAS, 1982). The significance of the differences among treatment groups was determined by Waller-Duncan k-ratio (Waller and Duncan, 1969). All statements of significance were based on probability of $P \leq 0.05$.

RESULTS

The present results showed that the water extract of papaya fruits contain 408.54 g/kg total phenolic however the ethanol extract contain 296.85 g/kg (Table 2). Moreover, the DPPH radical scavenging activity (SA) for these extracts recorded 32.73% and 18.87% for the water and ethanolic extracts, respectively (Table 2).

Table 2: Total phenolic content and DPPH radical scavenging activity of the water and ethanolic extract of papaya fruits

Parameter	Extract	Water extract (WE)	Ethanol extract (EE)
Total phenolic content (g/kg)		408.54 ± 12.38	296.85 ± 14.25
DPPH radical scavenging activity (%)		32.73 ± 2.67	20.75 ± 3.26

In the *in vivo* assay, WE and EE of papaya fruits were evaluated for their protective role against OTA-induced kidney

damage in rats. The experimental animals within different groups were appeared healthy and no mortality occurred in any of the treatment groups except only one rat died on day 18 in the group fed on OTA-contaminated diet. The results revealed that body weight gain (Fig. 1) and food intake (Fig. 2) were significantly decreased in animals fed OTA-contaminated diet compared to the control group. Food intake and body weight gain for the groups treated with either WE or EE were comparable to the control group. However, the administration of the water extract or the ethanolic extract plus OTA could induce a significant improvement in food intake and body weight gain.

The serum biochemical analysis (Table 3) revealed a significant increase in creatinine, urea, K^+ and NO accompanied with a significant decrease in Na^+ in the group fed OTA-contaminated diet *alone*. Animals received the extracts alone showed a significant decrease only in creatinine and were comparable to the control group regarding the level of urea and Na^+ however; K^+ showed a slight increase compared to the control group. Co-administration of WE or EE with OTA improved the kidney function indices towards the control values although they were still differ significantly than the control.

The effects of both extracts on antioxidant enzyme activities (GSH-Px and SOD), total antioxidant capacity, MDA and ATP-ase in kidney tissue (Table 4) showed that OTA administration induced a significant decrease in GSH-Px, SOD, TAC and ATP-ase parallel with a significant increase in MDA. Animals treated with WE or EE alone showed a significant increase in GSH-Px and SOD however; TAC level was within the normal range of the untreated control group. Moreover, MDA level was significantly decreased in these groups but ATP-ase was significantly decreased only in the group treated with WE. Treatment with WE or EE plus OTA showed a significant improve in all the biochemical parameters tested towards the control value and succeeded to normalize TAC in both groups and ATP-ase only in the group treated with WE plus OTA (Table 4).

The results of DNA fragmentation as determined by gel electrophoresis in the kidney tissue to assess the apoptotic cell death are presented in Fig (3). OTA-contaminated diet induced a significant increase in DNA fragmentation percentage reached 56.45%. However, animals treated with the extracts alone did not show any significant differences in DNA fragmentation. Administration of the extracts to the animals fed OTA-contaminated diet succeeded to induce a significant reduction in the elevation level of DNA fragmentation although these values were still higher than the control value.

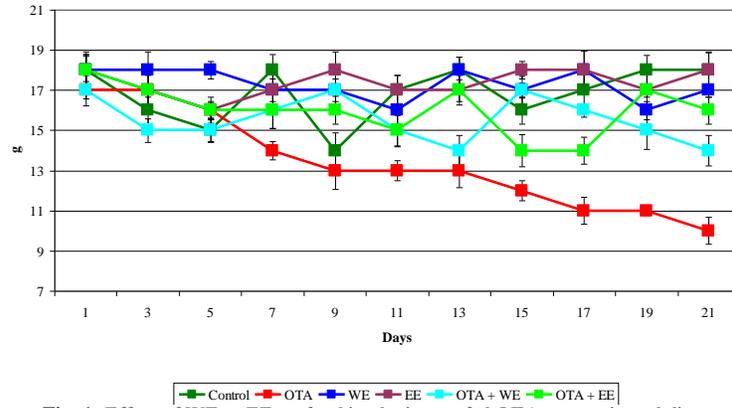


Fig. 1: Effect of WE or EE on food intake in rats fed OTA-contaminated diet

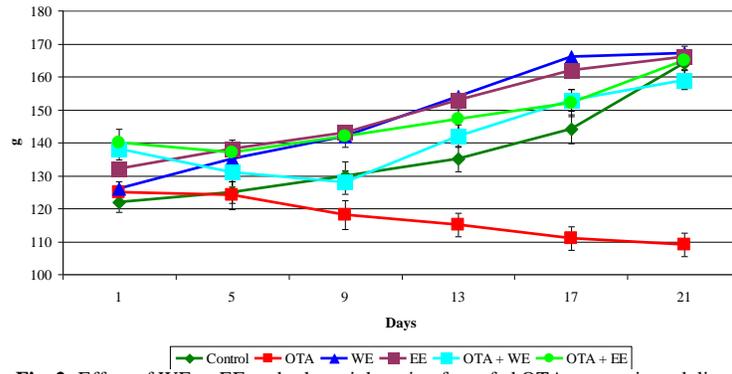


Fig. 2: Effect of WE or EE on body weight gain of rats fed OTA-contaminated diet

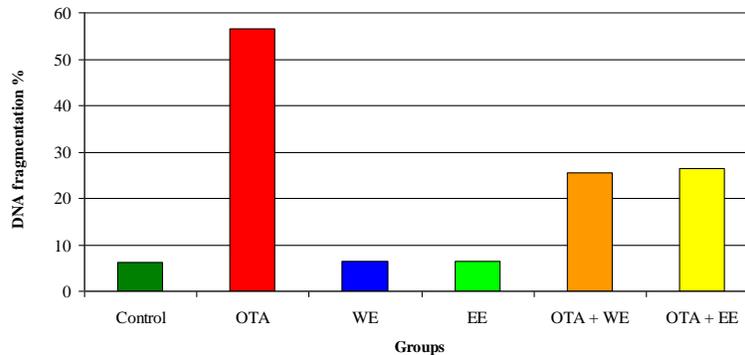


Fig. 3: Effect of WE or EE on DNA fragmentation in the kidney of rats fed OTA-contaminated diet

Table 3: Effect of water or ethanol extracts on serum biochemical parameters of kidney function in rats fed OTA-contaminated diet.

Parameter	Control	OTA	WE	EE	OTA + WE	OTA + EE
Creatinine (mg/dl)	0.82 ± 0.06 ^a	1.17 ± 0.04 ^b	0.79 ± 0.06 ^c	0.78 ± 0.05 ^c	0.81 ± 0.04 ^a	0.85 ± 0.04 ^d
Urea (mg/dl)	48.29 ± 2.58 ^a	87.41 ± 2.54 ^b	47.80 ± 2.96 ^a	48.16 ± 2.29 ^a	68.86 ± 2.09 ^c	65.05 ± 4.44 ^c
Na ⁺ (mmol/L)	48.56 ± 2.52 ^a	23.03 ± 2.62 ^b	47.53 ± 2.09 ^a	48.55 ± 3.41 ^a	42.17 ± 2.05 ^c	36.62 ± 1.28 ^d
K ⁺ (mmol/L)	5.34 ± 0.51 ^a	9.85 ± 0.35 ^b	6.03 ± 0.61 ^c	6.86 ± 0.43 ^c	8.21 ± 0.64 ^d	8.09 ± 0.56 ^d
NO (µmol/L)	78.23 ± 3.22 ^a	142.5 ± 5.32 ^b	59.43 ± 3.45 ^c	54.54 ± 3.41 ^c	95.34 ± 3.32 ^d	84.65 ± 4.11 ^e

Within each row, means superscript with different letter are significantly different (P ≤ 0.05).

Table 4: Effect of WE or EE of papaya fruits on GSH-Px, SOD, TAC, MDA and ATP-ase in the kidney of rats fed OTA-contaminated diet.

Parameter	Control	OTA	WE	EE	OTA + WE	OTA + EE
GSH-Px (unit/mg protein)	250.53 ± 4.23 ^a	122.73 ± 5.32 ^b	257.93 ± 7.26 ^a	262.56 ± 5.93 ^a	230.67 ± 7.56 ^c	233.78 ± 6.43 ^c
SOD (unit/mg protein)	237.93 ± 6.32 ^a	101.76 ± 5.83 ^b	243.73 ± 3.76 ^c	254.84 ± 6.65 ^d	225.76 ± 5.37 ^e	228.74 ± 8.53 ^e
TAC (µmol/g)	27.19 ± 0.06 ^a	17.14 ± 0.07 ^b	27.32 ± 0.06 ^a	27.48 ± 0.03 ^a	27.49 ± 0.06 ^a	27.37 ± 0.11 ^a
MDA (nmol/g)	53.99 ± 3.32 ^a	91.74 ± 2.81 ^b	37.97 ± 1.98 ^c	30.38 ± 1.02 ^d	32.05 ± 2.31 ^d	40.09 ± 6.19 ^e
Na ⁺ -K ⁺ -ATPase (µmol pi/hr/g)	339.22 ± 6.53 ^a	312.82 ± 2.20 ^b	331.84 ± 2.89 ^c	341.61 ± 5.18 ^a	339.76 ± 4.56 ^a	329.17 ± 4.40 ^d

Within each row, means superscript with different letter are significantly different (P ≤ 0.05).

The cytogenetic analysis showed that OTA induce a serious disturbance in the kidney genes expression. Rats fed OTA-contaminated diet showed a significant decrease in Cu-Zn SOD mRNA (Fig. 4) and GPx mRNA (Fig. 5) compared to the normal control group. Treatment with the extracts alone enhanced the gene expression of both enzymes and succeeded to induce a significant improvement in GPx mRNA and Cu-Zn SOD mRNA gene expression. Moreover, the best results were achieved in the group treated with EE. The histological examination of the kidney of control animals or the animals treated with WE or EE showed normal structure of glomeruli, renal tubules renal corpuscle (Fig. 6A). The kidney of animals treated with OTA showed degenerated renal tubules with hyaline casts and vacuolar degeneration in the tubular epithelial cells and hyaline casts in their lumen (Fig. 6B). The microscopic examination of the kidney tissue of rats treated with OTA plus WE showed that most of renal

tubules exhibit improvement except some tubules shows sclerosis, some glomeruli are normal and others are destructed (Fig. 6C). The kidney of rats fed OTA-contaminated diet and treated with EE showed nearly normal renal tubules, renal corpuscles and inter-tubular invasive inflammatory cells (Fig. 6D).

The histochemical examination of the kidney sections of the control rats stained with Bromophenol blue showed normal density of total protein in renal tubular epithelial cells (Fig. 7A). Animals treated with WE (Fig. 7B) or EE (Fig. 7C) showed normal distribution of bromophenol blue reaction suggested normal protein contents. The kidney of rats fed OTA-contaminated diet showed weak reaction in some tubules and mild reaction in the glomerular tufts (Fig. 7D). The kidney sections of rats treated with OTA plus WE (Fig. 7E) or EE (Fig. 7F) showed a strong reaction in the renal tubules and a moderate reaction in the glomeruli (Fig. 7E).

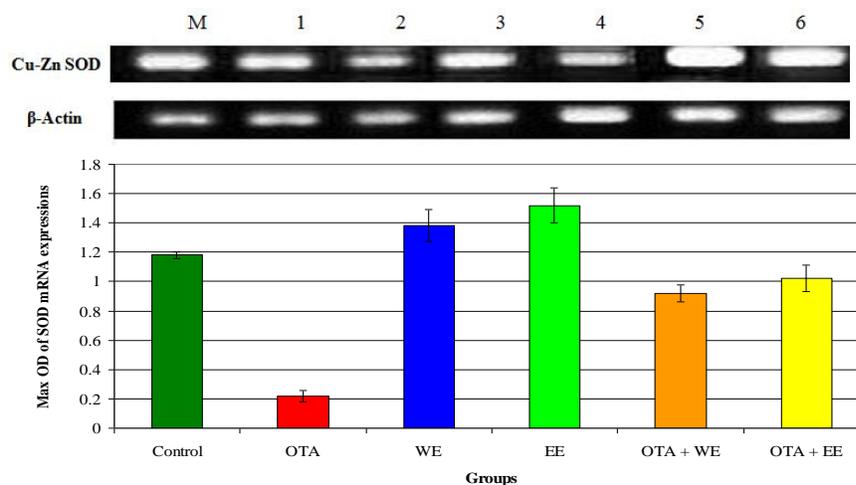


Fig. 4: Effect of WE and EE of papaya fruits on the ratio between Cu-Zn SOD/ β -actin mRNA in the kidney of rat fed OTA-contaminated diet.

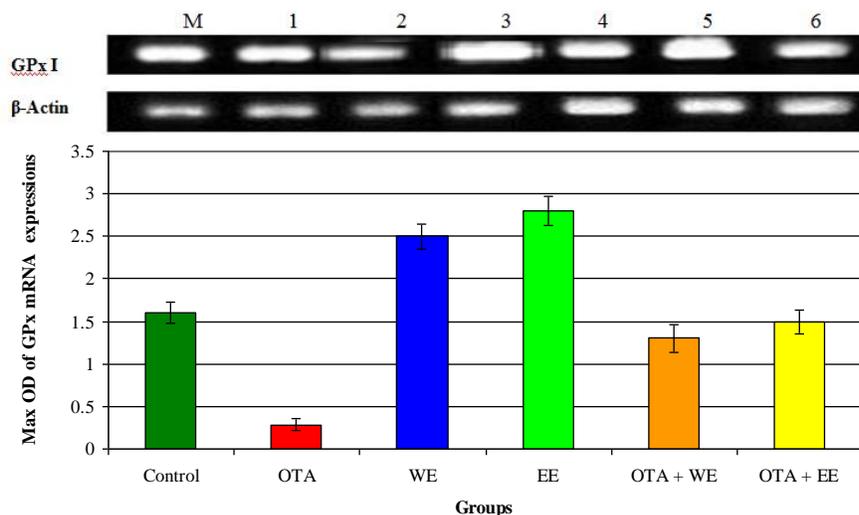


Fig. 5: Effect of WE and EE of papaya fruits on the ratio between GPx1/ β -actin mRNA in the kidney of rat fed OTA-contaminated diet.

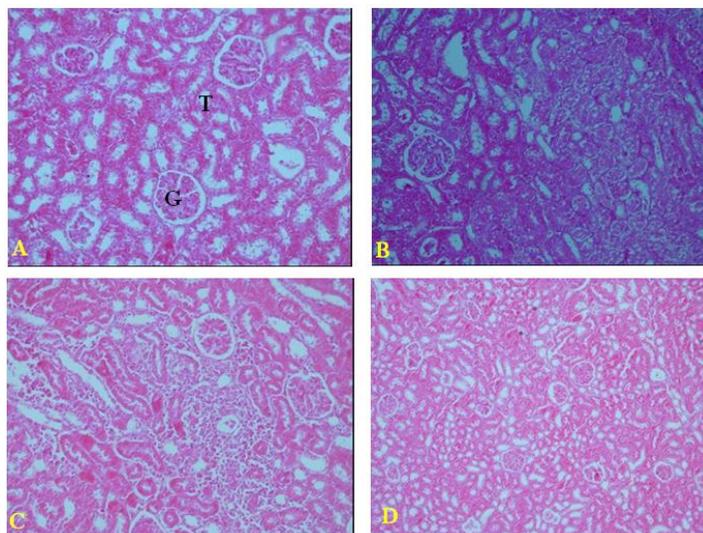


Fig. 6: A photomicrograph of a section in the kidney of (A) control rats or those treated with WE or EE showing normal structure of glomeruli (G), and renal tubules (T), (B) the kidney of rats fed OTA-contaminated diet showing degenerated renal tubules (in right side) with hyaline casts (in left side), (C) the kidney of rats fed OTA-contaminated diet and treated with WE showing improvement in most of renal tubules except some tubules shows sclerosis, some glomeruli are normal and others are destructed and (D) the kidney of rats fed OTA-contaminated diet and treated with EE showing nearly normal renal tubules and renal corpuscles and inter tubular invasive inflammatory cells.

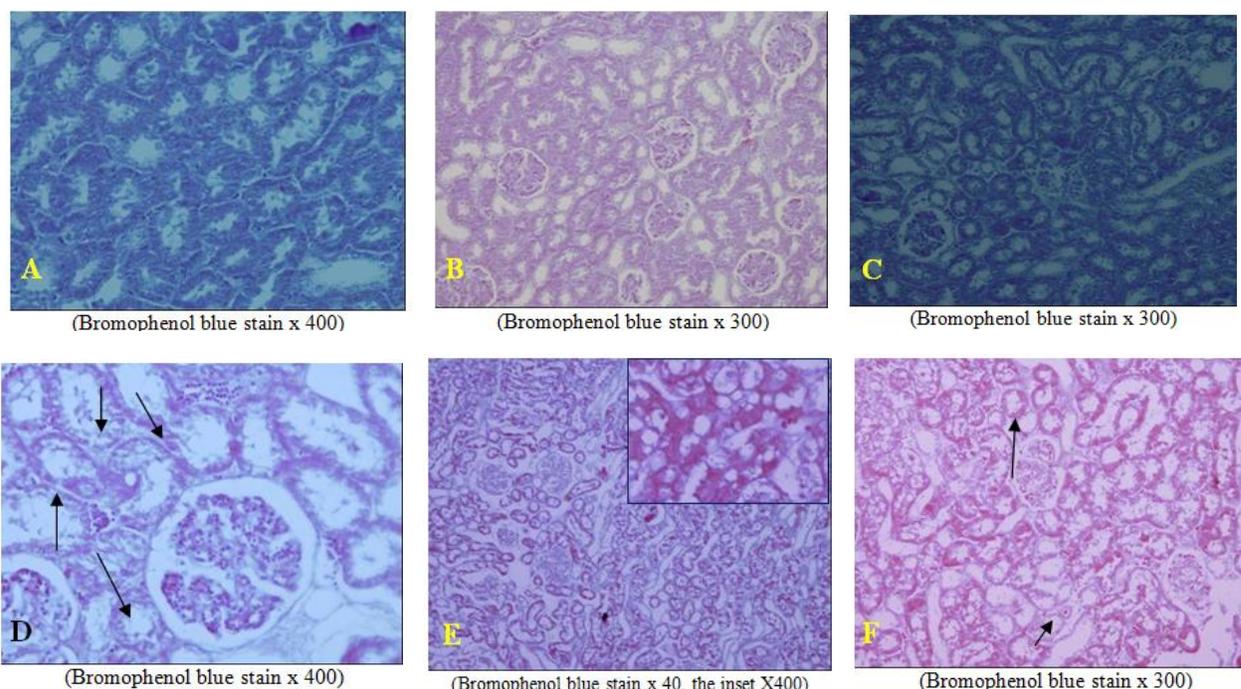


Fig. 7: A photo micrograph in kidney tissue of (A) control rat showing the normal density of total protein in renal tubular epithelial cells manifested by the blue color reaction, (B) rats treated with WE showing the normal distribution of bromophenol blue reaction, (C) rats treated with EE showing normal distribution of bromophenol blue reaction in different renal tissues, (D) rats fed OTA-contaminated diet showing the distribution of protein reaction in renal tubules, renal corpuscles and the hyaline casts revealed weak reaction in some tubules (arrow) and mild reaction in the glomerular tufts, (E) rats fed OTA-contaminated diet and treated with WE showing strong reaction in the renal tubules (inset) and a moderate reaction in the glomeruli and (F) rats fed OTA-contaminated diet and treated with EE showing a moderate reaction in the renal tubules have moderate reaction and mild reaction in the glomeruli.

DISCUSSION

In the current study, water extract of papaya fruits showed a higher content in total phenol than the ethanolic and both extracts have a potential DPPH radical scavenging activity but was

obvious in the water extract. In this concern, previous studies revealed that water extract of papaya fruits contains significant amounts of α -tocopherol (Ching and Mohamed, 2001), benzylisothiocyanate (Basu and Haldar, 2008), alkaloids such as carpasemine and carpain (Iyer *et al.*, 2011), lycopene (van

Breemen and Pajkovic, 2008), proteolytic enzymes such as chymopapain and papain (Seigler *et al.*, 2002), cystatin, ascorbic acid, cyanogenic glucosides and glucosinolates (Seigler *et al.*, 2002), triterpenes, organic acids (Cowan, 1989; Osuna-Torres *et al.*, 2005), as well as flavonoids (Miean and Mohamed, 2001). However, the ethanolic extract was found to be rich in sulfurous compounds (benzyl isothiocyanate) and recently Galang *et al.* (2016) isolated 5- β -Dglucopyranosyloxy-2-hydroxybenzoate and methyl β -D-glucopyranoside which showed significant DPPH radical scavenging capacity. According to Sancho *et al.*, (2011), the higher DPPH radical scavenging activity for the water extract is associated with the higher total phenolic content (Babu *et al.*, 2010).

In the *in vivo* assay, the water and ethanolic extract of papaya fruits were evaluated for their protective role against OTA-induced kidney damage in rats. The selected dose of OTA and papaya extracts were literature based (Abdel-Wahhab *et al.*, 2005; Rajkapoor *et al.*, 2002, respectively). Only one rat died on day 18 in the group fed on OTA-contaminated diet and showed signs of nephrotoxicity typical to those reported in the literature of ochratoxicosis (Malekinejad *et al.*, 2011; Qi *et al.*, 2014). The recorded decrease in body weight reported in the current study was primarily due to OTA and not to reduced food intake as indicated by our food restriction study. Similar to these observations, OTA was reported to induce a reduction in body weight gain and food intake in rats (Abbas *et al.*, 2013) and broilers chicken (Nedeljković-Trailović *et al.*, 2015; Sakthivelan and Rao, 2010).

The increase in creatinine and urea levels reported in the present study suggested the nephrotoxicity as reported earlier in different species of animals (Elaroussi *et al.*, 2007; Kumar *et al.*, 2008). The level of serum urea and creatinine is generally depending on their excretion through the glomerular filtration and the high level of creatinine is indicative of the impairment of kidney function (Malekinejad *et al.*, 2011). The increased level of these markers occurred when they accumulate proportionally with the number of the destroyed nephrons and hence reflect directly the functional situation of the kidneys (Cariddi *et al.*, 2016; Mir and Dwivedi, 2010). On the other hand the decreased levels of serum sodium and potassium reported herein suggested that OTA induced damages in the proximal tubules. It is well known that sodium presumably is reabsorbed regularly along the proximal and distal tubules (Bell *et al.*, 1968) however; potassium is reabsorbed in the proximal and secreted in the distal tubules (Malnic *et al.*, 1964; Pitts, 1968). Consequently, the decreased level of serum potassium indicated the impairment of its reabsorption during ochratoxicosis (Purchase and Theron, 1968; Huff *et al.*, 1975). It is well documented that NO exhibits a wide range of biological activities under the normal conditions; on the contrary, in excess production, it may lead to the formation of a toxic radical. Actually, NO react with O_2^- leading to the formation of the prooxidant peroxy nitrite $ONOO^-$ resulting in nitrosative stress (Radi, 2004). On the other hand, the significant increase in the level of NO reported in the current study in animals treated with OTA suggesting the ability of OTA to induce overproduction of

NO in the kidney and/or other organs (e.g. the liver) resulting in the elevation of nitrate and nitrite levels (Sorrenti *et al.*, 2012).

Na^+K^+ -ATP-ase is an enzyme located in the plasma membrane of all animal cells and helps to maintain the resting potential, the effective transport and the regulation of cellular volume (Hall *et al.*, 2006). In most animal cells, it act as a signal transducer/integrator to regulate mitogen-activated protein kinase (MAPK) pathway, ROS, intracellular calcium beside it is responsible for the ATP-dependent transport of potassium and sodium across the cell membrane (Kulkarni *et al.*, 2002). Moreover, Na^+K^+ -ATP-ase drives the transport of sugars and amino acids. In the present study, Na^+K^+ -ATP-ase activity was decreased in the animals fed OTA-contaminated diet suggesting the damage of cell membrane of the kidney and cell necrosis due to the oxidative stress. OTA was reported to have inhibitor effect on mitochondrial respiration which may be accounted for the impairment of renal function due to the depletion of ATP (Duarte *et al.*, 2009). Although several mechanisms were suggested for the mode of action of OTA but the strong ATP depleting effect seems to be the most pronounced (Ringot *et al.*, 2006).

OTA-induced oxidative stress is partially contributed to nephrotoxicity and carcinogenicity after a long term of exposure (Abdel-Wahhab *et al.*, 2008; Baldi *et al.*, 2004). In the current study, OTA also decreased the antioxidant enzymes GSH-Px and SOD and their gene expression as well as TAC accompanied with the elevation of MDA suggesting the conjugation between GSH-Px and OTA and/or its metabolites (Abdel-Wahhab *et al.*, 2005). Moreover, Smith-Kielland *et al.* (1986) reported that selenium is an essential cofactor for GSH-Px and selenium deficiency reduces the GSH-Px activity. Hence, OTA was suggested to interference with selenium absorption (Meki and Hussein, 2001). Taken together, the decreased in food intake reported herein and the interference of OTA with selenium absorption may be two reasons for the decreased activity of GSH-Px. On the other hand, SOD is a copper/ zinc-containing enzyme and is responsible for the catalytic dismutation of the highly reactive or potentially toxic superoxide radicals to hydrogen peroxide (Ozçelik *et al.*, 2004; Sutken *et al.*, 2007). In this concern, Meki and Hussein (2001) suggested that OTA interact with zinc and copper in SOD molecules and inhibits its activity and produces inhibition of the enzyme activity. In addition, OTA produces various changes in the cells such as the enhancement of the cell permeability to Ca^{2+} . The increase in Ca^{2+} concentration in the cell beside the presence of OTA as prooxidant lead to uncouple the oxidative phosphorylation consequently increase the electrons leakage from respiratory chain, generate O_2^- and hence H_2O_2 (Abdel-Wahhab *et al.*, 2005; Ozçelik *et al.*, 2004). This lack in the adequate supply of GSH and NAD(P)H permits the consumption of H_2O_2 by the NAD(P)H dependent GSR and GSH dependent GPx (Abdel-Wahhab *et al.*, 2008; Hohler *et al.*, 1997). Another mechanism for the generation of ROS by OTA is the increased of free iron concentration in the cell which stimulates the over production of OH through the Fenton-like reaction due to the mobilization of Fe^{2+} by Ca^{2+} (Abdel-Wahhab *et al.*, 2005, 2008).

The results of the present study also revealed that OTA administration induced a significant increase in the percentage of DNA fragmentation and the appearance of new bands in the animal fed OTA-contaminated diet. Although EFSA (2006) reported that OTA is not a mutagenic due to the absence of OTA-DNA adducts formation in rats, other strong evidences suggested that the toxicity of the kidney and the DNA damage are mainly attributable to the oxidative stress (Abdel-Wahhab *et al.*, 2008, 2016; Bouslimi *et al.*, 2008). According to Schaaf *et al.* (2002), the increase of ROS production, the decrease in antioxidants capacity and the elevation of oxidative DNA damage in proximal tubular cells may be due to ROS generation by OTA or the loss of cell viability (Mally *et al.*, 2005a,b). Moreover, the histological and histochemical results reported herein indicated that OTA induced severe changes in the renal tissues similar to those reported in the literature (Mansour *et al.*, 2011).

It is well documented that dietary intake of antioxidants is an effective and feasible way to boost and immunize endogenous defense systems due to their role as free radical scavenging activity resulting in the cytoprotection. In the current study, treatment with papaya extracts succeeded to ameliorate and/or counteract the oxidative stress of OTA. These results also suggested that the water extract was more effective than the ethanolic extract. According to Raj Kapoor *et al.* (2002), the LD50 of the papaya water extract was 2516.53 mg/kg while it was 2426.37 mg/kg for the ethanolic extract which suggest the effectiveness and the safety of the water extract. Moreover, both extracts succeeded to improve body weight gain and food intake as well as induced a significant reduction in MDA and restored all the other parameters to the normal clinical ranges reported previously (Vij and Prashar, 2015). The extract also succeeded to improve the histological picture of the renal tissue.

One of the possible mechanism(s) of the protective role of the extracts against OTA-induced nephrotoxicity may be the antioxidant and/or free radical scavenging activities due to their high content of flavonoids and alkaloids (Adeneye and Benebo, 2008; Miller and Rice-Evans, 1997). These reports are in agreement with the findings of the present results which indicate that the papaya extracts are rich in total phenolic compounds and have a potential DPPH radical scavenging activity *in vitro* and *in vivo* (Olabinri *et al.*, 2010). Moreover, polyphenols also are well known to have a wide range of biological activities including antioxidant and anti-inflammatory properties against oxidative stress and chronic inflammation (Siriwardhana *et al.*, 2013). Previous report indicated that the high content of flavonoid, tannins, alkaloid and saponin in papaya extract induce the profound *in vivo* and *in vitro* stabilizing activity on the lysosomes of laboratory animals (Oyedapo, 2001). Moreover, plant flavonoids such as tannins, saponins and serotonin show an antioxidant activity *in vitro* and *in vivo* and stabilize the erythrocyte membrane through the binding of cations and other biomolecules (Galang *et al.*, 2016; Santiago-Silva *et al.*, 2011). In this concern, Imaga *et al.* (2010) reported that the water extract of papaya has anti-tumor effect and suppressed the proliferative

responses of solid and haematopoietic tumor cell lines derived from different organs including breast and lung adenocarcinoma, cervical, hepatocellular and pancreatic epithelioid carcinoma and mesothelioma in a dose-dependent manner. According to Basu and Haldar (2008), other mechanisms for the protective role of papaya extracts against OTA- induce kidney damage via the enhanced production of Th1 type cytokines such as TNF- α , IFN- α , and IL-12, or through inducing a shift from Th2 to Th1 type immune response. Furthermore, Santiago-Silva *et al.* (2011) suggested that papaya fruits provide several functional activities due to the diversity of amines content consequently; it can be used for different nutritional needs. Taken together, the results of the *in vitro* and *in vivo* assay of the current study indicated that papaya extracts succeeded to a great extent to counteract the oxidative stress of OTA and to protect the kidney against its toxic effects.

CONCLUSION

The results of the present study concluded that the water and ethanolic extracts of papaya fruits have a potential antioxidant activity as indicated by the high concentration of total phenol and high DPPH radical scavenging activity *in vitro*. Treatment with OTA resulted in severe kidney injury as manifested by the disturbances in biochemical indices of kidney, the suppression of antioxidant gene expression and the increased DNA fragmentation percentage as well as the histological and histochemical changes in the kidney tissue. Both water and ethanolic extracts of papaya fruits showed a potential protective activity against the kidney injury induced by OTA through different mechanisms mainly their free radical scavenging properties and counteracting the free radical generation.

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