Grape seed and skin extract protects kidney from doxorubicin-induced oxidative injury

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Abstract: The study investigated the protective effect of grape seed and skin extract (GSSE) against doxorubicin-induced renal toxicity in healthy rats. Animals were treated with GSSE or not (control), for 8 days, administered with doxorubicin (20mg/kg) in the 4th day, and renal function as well as oxidative stress parameters were evaluated. Data showed that doxorubicin induced renal toxicity by affecting renal architecture and plasma creatinine. Doxorubicin also induced an oxidative stress characterized by an increase in malondialdehyde (MDA), calcium and H_2O_2 and a decrease in catalase (CAT) and superoxide dismutase (SOD). Unexpectedly doxorubicin increased peroxidase (POD) and decreased carbonyl protein and plasma urea. Treatment with GSSE counteracted almost all adverse effects induced by doxorubicin. Data suggest that doxorubicin induced an oxidative stress into rat kidney and GSSE exerted antioxidant properties, which seem to be mediated by the modulation of intracellular calcium.

Keywords: GSSE, Doxorubicin, kidney, calcium, antioxidant activities.

INTRODUCTION

Doxorubicin (Dox) is an anthracycline commonly used in the treatment of various malignant tumors such as breast cancer, soft tissue sarcoma, leukaemia, and childhood cancers (Hiroshi *et al.*, 2012). However, its administration has been largely restricted due to its multiple and multi-organ toxic side effects including kidney, heart and testis (Gillick *et al.*, 2002; Yilmaz *et al.*, 2006). Although the exact mechanism of Dox-induced nephrotoxicity remains unclear, it is believed to be underlied by an oxidative stress status through reactive oxygen species (ROS) formation, iron dependent oxidation of proteins and membrane lipid (Pritsos and Ma, 2000).

It has been suggested that the regular consumption of fruits and vegetables diminished the risk of certain malignancies (Tang *et al.*, 2014). Therefore, phytochemicals can be considered as promising chemopreventive agents. Interventional studies have demonstrated that oral administration of grape seed extract lowers ROS generation and plasma protein carbonylation, while enhancing the activity of the endogenous antioxidant system (Busserolles *et al.*, 2006; Balu *et al.*, 2005). The antioxidant effect of grape seed extract has even been confirmed in clinical trials (Rodr´yguez *et al.*, 2010). GSE has wide-ranging benefits including cardio-protective (Decordé *et al.*, 2009), reno-protective (Safa *et al.*, 2010) and hepato-protective (Sehirli *et al.*, 2008) properties. Grape seed extract also protects against cisplatin-induced nephrotoxicity in rat (Gao *et al.*, 2014), and azathioprine-induced hepatotoxicity in rats (El-Ashmawy *et al.*, 2010). GSE is a complex mixture of polyphenolics classified as flavonoid and non-flavonoid compounds (Khanal *et al.*, 2009). The most common flavonoids found in grapes are anthocyanins, flavonols, flavanols, dihydroflavonols (astilbin and engeletin) and proanthocyanidins. Anthocyanins are found only in red grape varieties. Recently, the strong beneficial health effect of grape flavonoids has been directly connected to the so-called "French Paradox" (Georgiev *et al.*, 2014).

Resveratrol is a multi-organ protective owing to its antioxidant (Kelen and Tepe, 2007) and anti-inflammatory properties (Kowalczyk *et al.*, 2010). Proanthocyanidins exert antineoplasic effects by cell cycle arrest and induction of apoptosis (Kaur *et al.*, 2008).

The objective of the present study was to investigate the putative protective effect of grape seed and skin extract (GSSE), against Dox-induced nephrotoxicity in healthy rat. The present study was also, undertaken to investigate the effect of GSSE on kidney function biomarkers. We also assessed renal lipoperoxidation and carbonylation, antioxidant status and intracellular mediators implicated in Dox-induced nephrotoxicity, as well as the protection offered by GSSE.

MATERIALS AND METHODS

Chemicals

Thiobarbituric acid (TBA); 2,6,-di-tert-butyl-4-hydroxy-toluene (BHT); trichloroacetic acid (TCA); hydrogen

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peroxide (H_2O_2); 2-methoxyphenol (gaiacol); bovine catalase and 4-(1-Hydroxy-2-methylamino-ethyl)-benzene-1,2-diol (epinephrine), 2,4,dinitrophenyl ydrazine (DNPH) were procured from Sigma-Aldrich Co (Germany).

Preparation of grape seed and skin extract

GSSE was processed from a grape cultivar (Carignan) of *Vitis vinifera* from northern Tunisia. Seeds and skin were dried and grounded separately with an electric mincer (FP3121 Moulinex) until a fine powder was obtained. Total phenolic content was determined by the folin-Ciocalteau colorimetric method (Singleton and Rossi, 1965), flavonoïds and condensed tannins according to Dewanto *et al.* (2002) and Sun *et al.* (1998) respectively (table 1). GSSE composition was established by HPLC-MS/MS analysis (table 2). Briefly liquid chromatography was performed using a Perkin Elmer system series 200 equipped with a binary micro-pump. The analyses were carried out on a C18 column (Zorbax Eclipse XDB-C18, 4.6×150mm, particle size 5µm). The mobile phase A was 0.1% formic acid in water and the mobile phase B was 0.1% formic acid in acetonitrile. Elution was performed at a flow rate of 1ml min^{-1} and an injection volume of 20µl. Tandem mass spectrometry (MS/MS) was carried out using a 3200 QTRAP mass spectrometer (Applied Biosystems/ MDS Sciex Forster city USA) equipped with an electrospray ionization (ESI) interface. Data were acquired and processed with Analyst 1.5.1 software. The detector was set in the negative ion mode. The ion trap mass spectrometer was operating in the m/z 50-1700 mass range.

Powder mixture containing grape seed (50%) and skin (50%) was dissolved in 10% ethanol in darkness, vigorously vortexed for 10min, centrifuged at 10 000g for 15 min at 4°C for debris elimination and the supernatant containing soluble polyphenols was used.

Animals and treatment

Female Wistar rats (195-220g) were used in accordance with the ethic committee of Tunis University for care and use of animals and in conformity with NIH guidelines (National Research council, 1985). They were maintained in animal facility at controlled temperature (22±2°C), a 12h light-dark cycle and provided with food and water ad libitum. Rats were randomly assigned to one of four groups of six animals each and daily intraperitoneally (IP) administered with either vehicle (C: 10% ethanol) or GSSE (500mg/kg *bw*), for 8 days. Dox dissolved in saline was IP injected at 20mg/kg *bw* as one single dose on day 4 till day 8. At the end of the treatment, animals were anesthetized with urethane (40mg/ml), their kidney collected, weighted, homogenized in PBS buffer pH 7.4 with an ultrathurax T25 homogenizator, centrifuged (10 min at 10000g, 4°C) and supernatant used for the determination of oxidative stress status.

Creatinine and urea measurement

Whole blood was collected by cardiac puncture into heparinized tubes. Plasma was processed after centrifugation at 1000g for 10min at 4°C and creatinine and urea determined using an auto-blood analyser, Coulter (National Institute of Nutrition, Tunis).

Lipoperoxidation

Kidney lipoperoxidation was evaluated by malondialdehyde (MDA) measurement. Briefly, kidney homogenates were added to BHT-TCA solution containing 1% BHT (m/v) dissolved in 20% TCA (m/v) and centrifuged at 1000g for 5 min at 4°C. HCl (0.5 N) and TBA (120mM in 26mM Tris) were added to the supernatant. The mixture was then heated at 80°C for 10 min. After cooling, absorbance was determined at 532 nm using a Smart Spec 3000 Bio-Rad UV-visible spectrophotometer (Germany). An extinction coefficient for the MDA-TBA complex of 1.56 10^5 $M^{-1}cm^{-1}$, was used to determine MDA levels (Draper and Hadley, 1990).

Table 1: Phenolics levels in carignan GSSE.

Phenolics	Seed	Skin
Total phenolics (mg/g extract)	67	51
Total Flavonoïds (mg/g extract)	16	14
Non flavonoïds (mg/g extract)	51	37
Condensed tannins (mg/g extract)	1.22	3.43
Total anthocyanins (µg/g extract)	0.997	0.962

Protein carbonylation

Oxidative damage to proteins was evaluated by quantifying protein carbonylation in kidney according to Levine *et al.* (1990). After proteins precipitation with 20% TCA and centrifugation at 11000g for 3min at 4°C (Beckman J20), pellet was dissolved in 10mM DNPH containing buffer. After 3 washings with ethanol-ethylacetate (1:1), pellet was dissolved in 20mM potassium phosphate (pH 2.3) containing 6 M guanidine HCl. Absorbance was measured at 366 nm using the molar extinction coefficient of 22000 $M^{-1}cm^{-1}$ and results expressed as µmol carbonyl residues/mg protein.

Protein determination

Total soluble proteins were determined by the Biuret method (Ohnishi and Barr, 1978).

Antioxidant enzyme activities

Spectrophotometric analyses were determined using Beckman DU 640B spectrophotometer. Catalase (CAT; E.C.1.11.1.6.) activity was determined by measuring the initial rate of H_2O_2 disappearance at 240nm (Aebi, 1984). An extinction coefficient of 40$mM^{-1}cm^{-1}$ for H_2O_2 was used to calculate CAT activity.

Peroxidase (POD; E.C.1.11.1.7.) activity was determined at 25°C using guaiacol as hydrogen donor. A reaction

Table 2: LC-MS/MS data of some phenolic compounds found in carignan GSSE

Compounds	m/z negative mode $[M-H]^-$	MS^2 fragment	Relative abundance (%)	
			Seed	Skin
Catechin	289	245/108.8/122.8	2.27	0.36
Epicatechin	289	245/108.8/122,8	2.85	0.37
Procyanidin dimmer	577	289.3/407.4	0.47	ND
Procyanidin trimer	865	577	ND	ND
Quercetin	301	150.8/120.9	0.64	0.47
Resveratrol	227	184.6/143	0.14	ND
Rutin	609.19	300.1	1.51	0.5
Vanillin	151.14	135.7/108.1	10.67	7.75
Gallic acid	169	124.7/78.9	50.3	32.77
P-coumaric acid	163	119/93	ND	0.38
Rosmarinic acid	359.2	160.8/197.1	ND	0.75
2,5-dihydroxybenzoïc acid	152.7	108.7/90.7	30.58	51.96
Caffeic acid	179	135	ND	2.8
Chlorogenic acid	353	191	ND	0.34
Ferulic acid	193	134/89	0.55	1.46

mixture containing 1ml final volume and containing 19mM H_2O_2 in 50mM phosphate buffer pH 7, 9mM guaiacol and 50µl of enzyme extract was used. The reaction was initiated by the addition of H_2O_2 and monitored by measuring the increase in absorbance at 470nm. Peroxidase activity was expressed in nmol of guaiacol oxidized per min. A molecular extinction coefficient of $26.2mM^{-1}$ was used for calculation (Chance and Maehly, 1955).

Superoxide dismutase (SOD; E.C.1.15.1.1.) activity was measured by using modified epinephrine assay (Misra and Fridovich, 1972). At alkaline pH, superoxide anion O_2^- causes the auto-oxidation of epinephrine to adenochrome. Competing with this reaction, SOD inhibited the adenochrome formation. One unit of SOD is defined as the amount of extract that decreases the rate of adenochrome formation by 50%. Enzyme extract was added in 2ml reaction mixture containing 20µl epinephrine (5mg/ml), 10µl bovine catalase (0.4U/µl), and 62.5mM sodium carbonate/sodium bicarbonate buffer pH 10.2. Absorbance was determined at 480 nm. SOD isoforms were determined using KCN (3mM), which inhibited Cu/Zn-SOD or H_2O_2 (5mM) affecting both Cu/Zn-SOD and Fe-SOD, whereas, Mn-SOD was insensitive to both inhibitors.

Free iron measurement

Kidney free iron level was determined according to Leardi *et al.* (1998) using a commercially available kit from Biomaghreb, Tunisia. At acidic pH 4.8 all Fe^{3+} released from transferrine was reduced by ascorbic acid into Fe^{2+}, which constituted with ferrozine a purple colourful complex measurable at 560nm.

H_2O_2 content

Renal H_2O_2 was determined enzymatically according to Kakinuma *et al.* (1979), using a commercially available kit from Biomaghreb. Briefly, in the presence of peroxidase, H_2O_2 reacts with 4-amino-antipyrine and phenol to give a red colored quinoeimine which absorbed at 505nm. Results are expressed as mmol H_2O_2/mg protein.

Calcium measurement

Kidney ionizable calcium was determined according to Stern and Lewis (1957) using a commercially available kit from Biomaghreb. At basic pH calcium constituted with cresolphtalein a purple colourful complex measurable at 570 nm. Briefly, renal homogenates were added to reaction mixture containing 2-amino-2-methyl 1-propanol buffer (500mmol/L), cresolphtalein (0.62 mmol/L) and hydroxy-8 quinoleine (69mmol/L). Incubation was carried out at room temperature for 5 min assuming the complex stability for 1 hour.

Histopathological study

A piece of kidney was collected and fixed in 4% (v/v) formalin, dehydrated with alcohols and embedded in paraffin. 4µm sections were stained with hematoxylin and eosin (H&E) and examined in a blinded fashion under light microscope. From each group, one representative micrography of kidney tissue is shown (n=6).

STATISTICAL ANALYSIS

All statistical analyses were realized by unpaired Student's t-test or one-way analysis of variance (ANOVA). Data were expressed as means ± standard error of the mean (S.E.M.). All statistical tests were 2-tailed, and $p<0.05$ considered significant.

RESULTS

Composition of grape seed and skin extract

Phenolics levels found in seed and skin powder from carignan cultivar are shown in table 1. Total phenolics and

flavonoïds are slightly higher into seeds than skins, whereas condensed tannins are much represented into skins (3 fold). Among the 15 phenolic compounds identified within seed and skin, only 2,5 dihydroxybenzoïc, caffeic and ferulic acid were more abundant into skins than seeds (table 2).

Plasma creatinine and urea

We evaluated plasma creatinine and urea levels (fig. 1). Dox significantly increased plasma creatinine (+50%) (fig. 1A) but unexpectedly decreased urea (-25%) (fig. 1B). Pre-treatment with GSSE counteracted Dox-induced disturbances in these parameters to near control level.

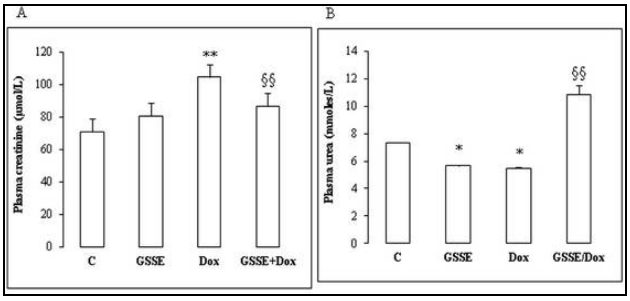

Fig. 1: Effect of Dox and GSSE on plasma creatinine and urea. Rats were pre-treated or not with GSSE during 8 days and challenged with a single dose of Dox in the fourth day. Plasma creatinine (fig. 1A) and urea (fig. 1B) were determined. Results are expressed as mean ± SEM (n=6). **indicated $p<0.01$ vs C. §§ indicated $p<0.01$ vs Dox.

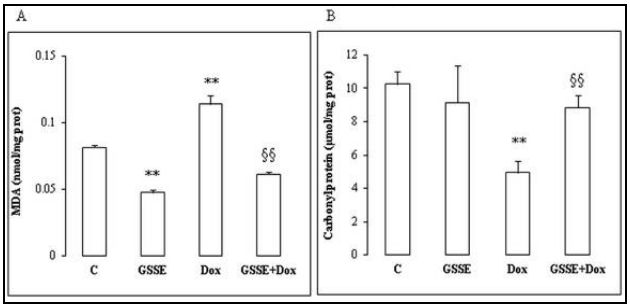

Fig. 2: Effect of Dox and GSSE on kidney oxidation. Rats were pre-treated or not with GSSE during 8 days and administered with a single dose of Dox at the fourth day. Kidney MDA (fig. 2A) and protein carbonylation (fig. 2B) were determined. Results are expressed as mean ± SEM (n=6). ** indicated $p<0.01$ versus C. §§ indicated $p<0.01$ versus Dox.

Renal lipoperoxidation and carbonylation

We reported in fig. 2 the effect of Dox and GSSE on kidney lipoperoxidation and carbonylation. Dox highly increased kidney MDA (+40%) (fig. 2A) but unexpectedly decreased protein carbonylation (-50%) (fig. 2B) whereas, GSSE prevented the Dox- induced effects in lipoperoxidation and carbonylation.

Kidney antioxidant enzyme activities

We further asked whether Dox affected renal antioxidant enzymes. Dox decreased CAT (-25%) (fig. 3A) and SOD activity (-20%) (fig. 3C), but increased POD (+250%) (fig. 3B). GSSE abrogated almost all Dox-induced effect on antioxidant enzyme activities to near control level.

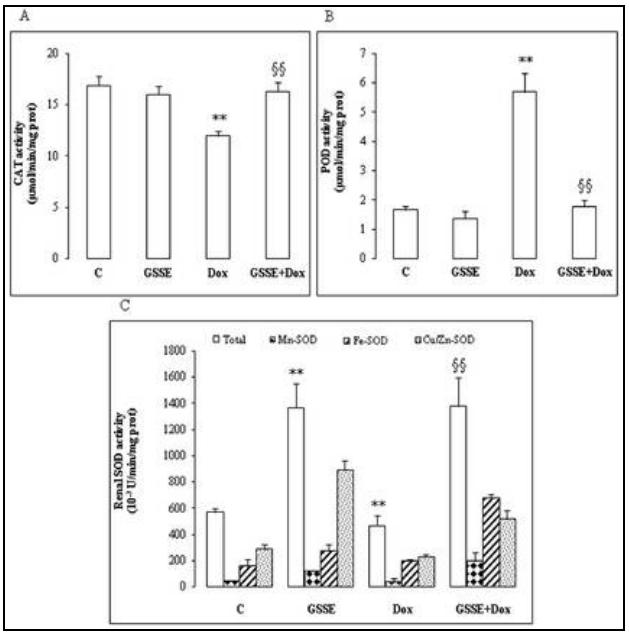

Fig. 3: Effect of Dox and GSSE on kidney antioxidant enzyme activities. Rats were pre-treated or not with GSSE during 8 days and treated with a single dose of Dox at the fourth day. Renal CAT (fig. 3A), POD (fig. 3B) and SOD (fig. 3C) activities were determined. Results are expressed as mean ± SEM (n=6). ** indicated $p<0.01$ versus C. §§ indicated $p<0.01$ versus Dox.

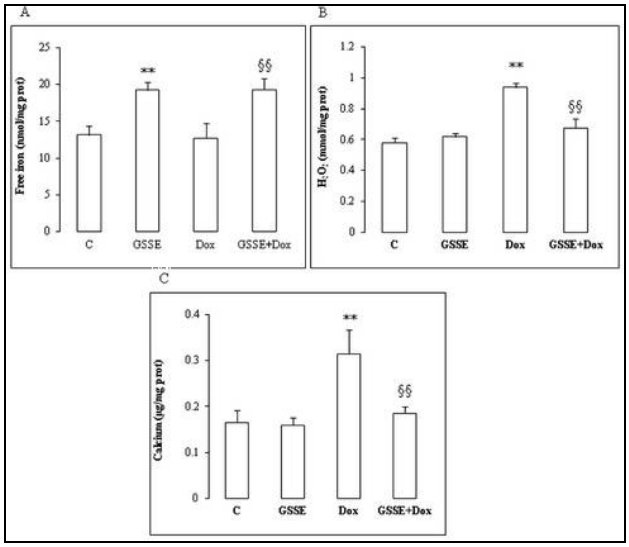

Fig. 4: Effect of Dox and GSSE on kidney free iron, H_2O_2 and calcium. Rats were pre-treated or not with GSSE during 8 days and challenged with a single dose of Dox at the fourth day. Kidney free iron (fig. 4A), H_2O_2 (fig. 4B) and calcium (fig. 4C) were determined. Results are

Table 3: Kidney index

	C	GSSE	Dox	GSSE+Dox
Initial body weight (g)	202.6±5.97	198,6±5,26	197±9.45	207.25±6.89
Final body weight (g)	232±3.44	31±12.80	157.66±6.88	156±6.55
Kidney index	69.51±2.4	69.42±2.53	87.01±3.91	98.51±4.05

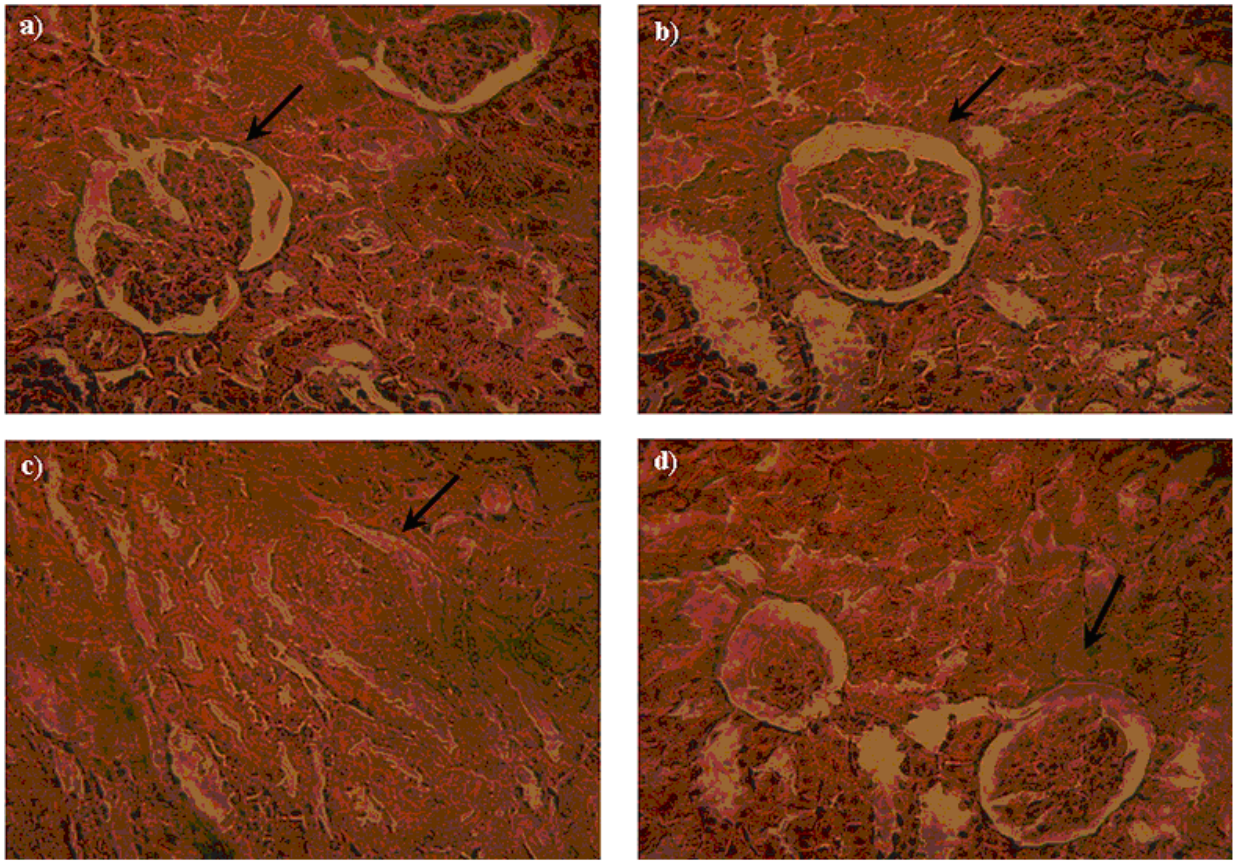

Fig. 5: Effects of Dox and GSSE on kidney histology in rats. Light microscopy of kidney tissue (HE stained kidney sections, 400×) were realised on Control (fig. 5A), GSSE (fig. 5B), Dox (fig. 5C) and GSSE + Dox (fig. 5D) groups.

expressed as mean ± SEM (n=6). **indicated $p<0.01$ versus C. §§ indicated $p<0.01$ versus Dox.

Kidney intracellular mediators

Fig. 4 deals with the effect of Dox and GSSE on some intracellular mediators. Dox had no effect on free iron (fig. 4A) but increased H_2O_2 (+62%) (fig. 4B) and ionizable calcium (+90%) (fig. 4C). Treatment with GSSE counteracted Dox-induced intracellular mediators disturbances to near control level.

Kidney index and histopathology

Dox-induced renal toxicity was assessed by increased kidney index from 69.5±2.4 (control) to 87.01±3.91 (Dox). GSSE+Dox treated animals exhibited increased kidney index (98.51±4.05) (table 3).

The histopathological study showed a Dox-induced alteration of the typical renal architecture (fig. 5c) when compared to control (fig. 5a). However, Dox-induced renal morphology alteration was alleviated by GSSE (fig. 5d), Whereas, GSSE on its own had no obvious effect on renal architecture (fig. 5b).

DISCUSSION

Dox is an anthracycline antibiotic widely used to treat cancers whose clinical use is restricted because of its serious side effects in particular renal injury. The present work deals with the renal toxic side effects of Dox in healthy rat and mainly shows that Dox altered renal function materialized by a significant increase in plasma creatinine and kidney index. Dox also induced an intra-renal oxidative stress as assessed by high lipoperoxidation level, low CAT and SOD activity and elevated H_2O_2 and calcium. Our data are in agreement with several previous works dealing with Dox-induced injury to non-targeted or safe tissues (for review see Carvalho *et al*., 2009), which

is believed to be mediated through oxidative burden (Rashikh *et al*., 2013). In this respect, the semiquinone form of the anthracycline was previously shown to play a key role in Dox-induced nephrotoxicity (Bachur *et al*., 1979) by means of O_2^- generation (Deman *et al*., 2001). However, our data also rise some discrepancies as for instance concerning Dox-induced increase in POD activity. Such result could be explained by Dox-induced translational modification (Rhee *et al*., 2005) inducing a gain in function as found in the present case for peroxidase activity. These data are reminiscent of the recent work of Sadi *et al*. (2012), on the differential effect of streptozotocin on post-translational modification of antioxidant enzymes as CAT, GPx and SOD into the kidney.

Other features of renal toxicity induced by Dox treatment are characterized by an elevation in H_2O_2 and a burst in Ca^{2+}. Intriguingly, no effect on free iron was detected, as Dox is currently believed to act via iron-dependent oxidative damage of proteins and lipids, nor did we have detected any effect on protein carbonylation. It is tempting to speculate that Dox-induced protein carbonylation is iron-dependent and that Dox-induced lipoperoxidation is not. Furthermore we found that Dox-induced calcium overload within the kidney, which is in accordance with most reported studies (Kalivendi *et al.*, 2005; Kim *et al.*, 2006), but in discordance with our previous work on Dox-induced cardiotoxicity, which was found to involve calcium depletion instead of calcium overloading (Mokni *et al*., 2012).

The most relevant result drawn from the current study is the reno-protection offered by GSSE. GSSE abrogated almost all Dox-induced renal disturbances as oxidative stress, calcium burden and morphological alterations. Prevention of Dox nephrotoxicity is of utmost importance. It has been achieved using various protective compounds such as deferoxamine (Bulucu *et al*., 2008), melatonin (Hrenák *et al*., 2013) resveratrol (Oktem *et al*., 2012) or quercetin (Heeba and Mahmoud, 2014). To our knowledge our data are the first to demonstrate a potent protection of GSSE against Dox-induced nephrotoxicity. Such protection has been obtained at high dosage GSSE i-e 500mg/kg *bw*, if we consider the weight of the starting grape seed powder. In our case, high dosage GSSE reaching 4g/kg, were highly safe when used in various experimental settings as obesity (Charradi *et al*., 2011), chemotherapy (Mokni *et al*., 2012) or after cerebral I/R (Safwen *et al*., 2014).

We do not yet know which GSSE component is at the basis of such nephro-protection, keeping in mind the complex composition of GSSE (Khanal *et al*., 2009) and also a likely synergism between numerous GSSE containing polyphenols.

In conclusion, high dosage GSSE exerted potent protection against Dox-induced renal toxicity in healthy rat and constitutes a potent and safe adjuvant in chemotherapy protocols.

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