Efficacy of grape seed and skin extract against doxorubicin-induced oxidative stress in rat liver

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Abstract: Doxorubicin (Dox) is an anthracycline used in chemotherapy, although it causes toxicity and oxidative stress. Grape seed and skin extract (GSSE) is a mixture of polyphenolic compounds with antioxidant properties. To evaluate the hepato-toxicity of Dox on healthy rats as well as the protective effect of GSSE, rats were treated with GSSE (500mg/kg *bw*) during 8 days. At the 4th day of treatment, they received a single dose of Dox (20 mg/kg *bw*). After the treatment (9th day), livers were collected and processed for oxidative stress status. Dox increased MDA (+ 900%), decreased catalase (-60%) and increased peroxidase (+90%) and superoxide dismutase (+100%) activities. In this latter case Dox mainly increased the iron isoform. Furthermore Dox altered intracellular mediators as catalytic free iron (-75%), H_2O_2 (-75%) and calcium (+30%). Dox also affected liver function by elevating plasma triacylglycerol and transaminases and liver morphology by altering its typical architecture. Importantly all Dox-induced liver disturbances were alleviated upon GSSE treatment. Dox induced liver toxicity and an oxidative stress mainly characterized by increased lipoperoxidation but not protein carbonylation. GSSE efficiently protected the liver from Dox-induced toxicity and appeared as a safe adjuvant that could be incorporated into chemotherapy protocols.

Keywords: GSSE, Dox, liver, TBARS, free iron, antioxidant enzymes.

INTRODUCTION

Doxorubicin (Dox), an anthracycline antibiotic isolated from *Streptomyces peacetius* has been used for more than 30 years in the treatment of various carcinomas (Minotti *et al.*, 2004). However its clinical use is limited by its toxic side effects (Quiles *et al.*, 2006), which appear within minutes after infusion and may develop several weeks or months after the recurrent administration of the drug. They can include nausea, vomiting, myelo-suppression and also multi-organ dysfunction.

Grape seed and skin extract (GSSE) is a mixture of polyphenolic compounds displaying numerous beneficial health effects (for review see Nassiri-Asl and Hosseinzadeh, 2009). GSSE has been considered a potential protector against cardio toxicity induced by Dox chemotherapy (Quiles *et al.*, 2002; Bagchi *et al.*, 2002). These protective effects of GSSE were closely attributed to its iron chelating property (Kaiserová *et al.*, 2007). However studies on the protective role of GSSE against Dox-induced liver toxicity are scarce.

We aimed to determine the protective effect of GSSE on Dox-induced liver damage in rat. We found that pre-treatment with GSSE efficiently suppressed hepato-toxicity and oxidative stress induced by Dox. These effects are partly linked to free iron and calcium modulating activities of GSSE.

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MATERIALS AND METHODS

Chemicals

2-thiobarbituric acid (TBA; CAS number 504-17-6); 2,6,-di-tert-butyl-4-hydroxy-toluene (BHT; CAS number 128-37-0); trichloroacetic acid (TCA; CAS number: 76-03-9); hydrogen peroxide (H_2O_2; CAS number 7722-84-1); 2-methoxyphenol (gaiacol; CAS number: 90-05-1); bovine catalase (CAS number: 9001-05-2), 4-(1-Hydroxy-2-methylamino-ethyl)-benzene-1,2-diol (epinephrine; CAS number: 8060-13-7) and 2,4,dinitrophenyl hydrazine (DNPH; CAS number: 119-26-6) were obtained from Sigma-Aldrich (Germany). Doxorubicin hydrochloride was purchased from Pharmacia Italia (Italy).

Preparation and composition of GSSE

GSSE was prepared from a Carignan-grape cultivar of *Vitis vinifera* from northern Tunisia. Seeds and skins were dried and grounded separately with an electric mincer (FP3121 Moulinex) until a fine powder was obtained. Total phenolic content was determined by the colorimetric method of Folin-Ciocalteau (Singleton and Rossi, 1965). Flavonoids and condensed tannins were determined 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 high performance 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 × 150 mm,

particle size 5 µm). 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 50% seed and 50% skin was dissolved in 10% ethanol in the dark, vigorously vortexed for 10 min, centrifuged at 10 000 g for 15 min at 4°C for debris elimination and the supernatant containing soluble polyphenols was used.

Animals and treatment

In these experiments, female Wistar rats (220-240g) were used in accordance with the ethic committee of Tunis University for care and use of animals in conformity with NIH guideline (National Research Council, 1985). They were maintained in animal house at fixed temperature of 22±2°C with a 12 h light–dark cycle and provided with food and water ad libitum. Rats were randomly divided into four groups of six animals each and daily intraperitoneally (*ip*) injected for 8 days with vehicle (C: 10% ethanol), GSSE (500 mg/kg *bw*), Dox (20 mg/kg *bw*) or GSSE+Dox. Dox was dissolved in saline and administered as a single dose on day 4 till day 8. At the end of the treatment, rats were anesthetized with urethane (40 mg/ml), sacrificed and the liver collected, weighted and homogenized in PBS buffer pH 7.4 with an ultrathurax T25 homogenizator. After centrifugation (10 min at 10 000g, 4°C) supernatant was used for the determination of free iron, Ca^{2+}, H_2O_2, lipoperoxidation, protein carbonylation and antioxidant enzyme activities. Blood was also collected by cardiac puncture into heparinized tubes and processed for plasma determination of lipidemia and liver function parameters.

Lipidemia and liver function

Total cholesterol was determined into plasma and liver using a commercial kit from Sigma based on a modification of the cholesterol oxidase method of Allain *et al.* (1974). Plasma and liver triacylglycerol were measured enzymatically using the lipase-glycerol phosphate oxidase method of Mc Growan *et al.* (1983), with a commercial kit from Sigma. Plasma LDH was determined according to the method of Howell *et al.* (1997) using a commercial kit from Biomaghreb, Tunisia. Plasma transaminases (AST, ALT) were determined using a commercial kit from Biomaghreb according to the manufacturer's instructions (Reitman and Frankel, 1957).

Liver oxidative stress

Liver lipoperoxidation was determined by malondialdehyde (MDA) measurement (Draper and Hadley, 1990). Absorbance was determined at 532 nm and MDA concentration calculated using the absorbance coefficient of TBARS-TBA complex: 1.56×10^5 $cm^{-1}M^{-1}$. Oxidative damage to proteins was evaluated by quantifying protein carbonylation according to Levine *et al.* (1990). Absorbance was determined at 366 nm using the molar extinction coefficient of 22000 $M^{-1}cm^{-1}$ and results expressed as nmol carbonyl residues/mg protein.

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

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

H_2O_2 was determined enzymatic ally 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 505 nm. Results were expressed as mmol H_2O_2/mg protein.

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

Liver antioxidant enzyme activities were determined spectrophotometrically using a Beckman DU 640B spectrophotometer. Catalase (CAT) activity was determined at 240 nm, by measuring the initial rate of H_2O_2 disappearance (Aebi, 1984). CAT activity was calculated using the extinction coefficient of 40 $mM^{-1}cm^{-1}$ for H_2O_2.

Peroxidase (POD) activity was measured at 25°C using guaiacol as hydrogen donor. The reaction was initiated by the addition of H_2O_2 and monitored by measuring the increase in absorbance at 470 nm (Chance and Maehly 1955). POD activity was expressed as nmol guaiacol oxidized per min with a molecular extinction coefficient of 26.2 $mM^{-1}cm^{-1}$for calculation.

Table 1: Phenolic 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

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

Table 3: Liver and plasma lipidemia

		C	GSE	Dox	GSE+Dox
Plasma	Cholesterol (mmol/l)	0.97±0.02	1.11±0.02	1.39±0.06*	0.91±0.12
	Triacyglycerol (mmol/l)	1.08±0.18	0.99±0.18	0.22±0.01*	0.41±0.04*
Liver	Cholesterol (mmol/l)	16.25±1.17	25.16±2.88*	16.86±1.14	23.55±4.83*
	Triacylglycerol (ug/mg protein)	79.45±3.80	72.87±1.88	69.95±1.89	64.81±1.69

Superoxide dismutase (SOD) activity was determined according to Misra and Fridovich (1972) using a modified epinephrine assay. One unit of SOD was defined as the amount of extract that inhibited the rate of aden chrome formation by 50%, at 480nm. Characterization of the SOD isoforms was performed according to Loukili *et al*. (1999) using KCN (3mM), which inhibited Cu/Zn-SOD, or H_2O_2 (3mM) affecting both Cu/Zn-SOD and Fe-SOD whereas Mn-SOD was insensitive to both inhibitors.

Histopathological study

A piece of liver was cut at 0.5µm, mounted on slides, stained with hematoxylin and eosin (H&E) and examined in a blinded fashion under light microscope. One representative micrography of liver tissue from each group is shown (n=6).

STATISTICAL ANALYSIS

Unpaired Student's t-test or one-way analysis of variance (ANOVA) were used to analyze data and results expressed as means ± standard error of the mean (S.E.M.). All statistical tests were 2-tailed, and significance was considered for $p<0.05$.

RESULTS

Grape seed and skin extract composition

Phenolic 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 more represented into skins (3 fold). Among the 15 phenolic compounds identified within seeds and skins, only 2,5 dihydroxybenzoïc, caffeic and ferulic acid were more abundant into skins than seeds (table 2).

Plasma and liver lipids

We reported in table 3 the effect of Dox on plasma and liver cholesterol and triacyglycerol. Dox increased cholesterol (+ 44%) and decreased triacylglycerol (- 80%) into plasma but had no effect on liver lipids. GSSE counteracted the Dox-induced lipid disturbances till control level.

Plasma LDH and transaminases

We further evaluated plasma LDH and transaminases (fig. 1). Dox had no effect on plasma LDH (fig. 1A) but significantly increased by 56% and 68% plasma ALT and AST respectively (fig. 1B and 1C). Treatment with GSSE corrected the Dox-induced disturbances of transaminases to near control level.

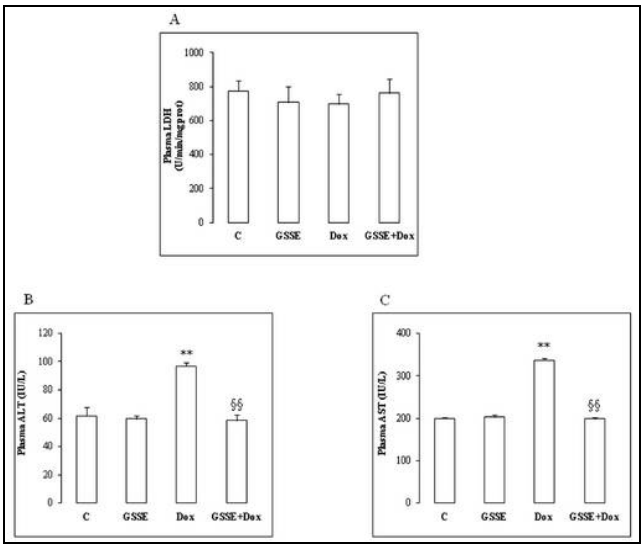

Fig. 1: Effect of Dox and GSSE on plasma LDH and transaminases**.**

Rats were pre-treated or not with GSSE during 8 days and challenged with a single dose of Dox at the fourth day. Plasma LDH (Fig. 1A), ALAT (Fig. 1B) and ASAT (Fig. 1C) were determined. Results are expressed as mean ± S.E.M. (n=6); ** indicated p<0.01 vs. C; §§ indicated p<0.01 vs. Dox.

Liver lipoperoxidation and carbonylation

We reported in fig. 2 the effect of Dox and GSSE on liver lipoperoxidation and protein carbonylation. Dox highly increased liver MDA (+ 900%) (fig. 2A) but had no effect on carbonylation (fig. 2B) and once again GSSE abrogated the pro-oxidative effect of Dox.

Liver antioxidant enzyme activities

We further asked whether Dox affected liver antioxidant enzyme activities. Dox decreased CAT (-60%) (fig. 3A) but increased POD (+90%) (fig. 3B) and SOD activities (+100%) (fig. 3C) mainly the Fe isoform (+200%) in this latter case. GSSE abrogated all Dox-induced alterations in antioxidant enzyme activities till control.

Liver intracellular mediators

Fig. 4 showed the effect of Dox and GSSE on liver intracellular mediators. Dox decreased free iron (-75%) (fig. 4A) and H_2O_2 (-75%) (fig. 4B) but increased ionizable calcium (+30%) (fig. 4C). GSSE counteracted the Dox-induced intracellular mediators disturbances to control level.

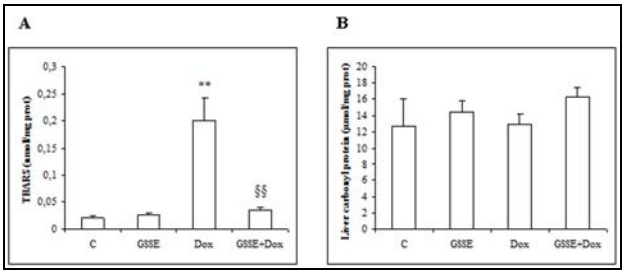

Fig. 2: Effect of Dox and GSSE on liver oxidation.

Rats were pre-treated or not with GSSE during 8 days and challenged with a single dose of Dox at the fourth day. Liver TBARS (*Fig. 2A*) and protein carbonylation (*Fig. 2B*) were determined. Results are expressed as mean ± S.E.M. (n=6); ** indicated p<0.01 *vs*. C; §§ indicated p<0.01 *vs*. Dox.

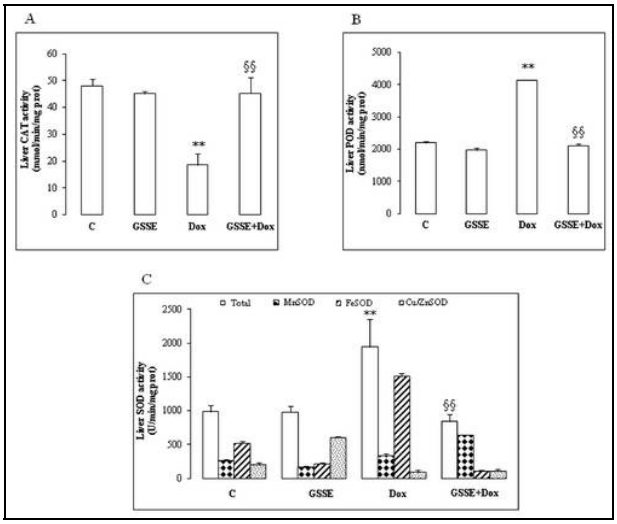

Fig. 3: Effect of Dox and GSSE on liver antioxidant enzyme activities.

Rats were pre-treated or not with GSSE during 8 days and challenged with a single dose of Dox at the fourth day. Liver CAT (*Fig. 3A*), POD (*Fig. 3B*) and SOD (*Fig. 3C*) activities were determined. Results are expressed as mean ± S.E.M. (n=6); ** indicated p<0.01 *vs*. C; §§ indicated p<0.01 *vs*. Dox.

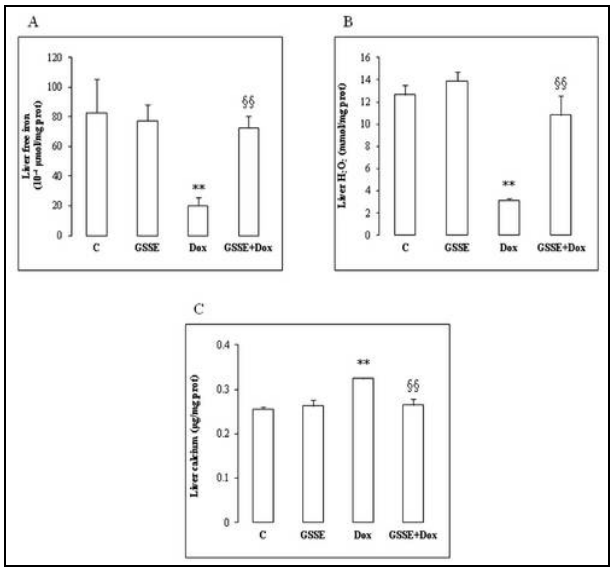

Fig. 4: Effect of Dox and GSSE on liver intracellular mediators.

Rats were pre-treated or not with GSSE during 8 days and challenged with a single dose of Dox at the fourth day. Liver free iron (*Fig. 4A*), H_2O_2 (*Fig. 4B*) and ionizable calcium (*Fig. 4C*) were determined. Results are expressed as mean ± S.E.M. (n=6); ** indicated $p<0.01$ *vs*. C; §§ indicated $p<0.01$ *vs*. Dox.

Liver histopathology

Histopathological study (fig. 5) showed that Dox altered the typical hepatic architecture as assessed by swollen hepatocytes with marked vacuolization and congestion (fig. 5C). GSSE treatment reversed the altered morphology to near normal architecture (fig. 5D).

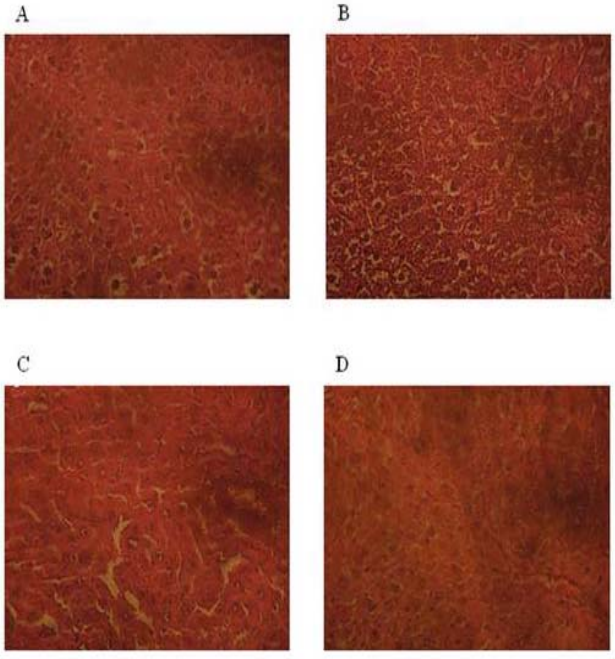

Fig. 5: Effect of Dox and GSSE on liver histology.

Rats were pre-treated or not with GSSE during 8 days and challenged with a single dose of Dox at the fourth day. Liver sections from control (*Fig. 5A*), GSSE (*Fig. 5B*), Dox (*Fig. 5C*) and GSSE + Dox (*Fig. 5D*) rats are shown (HE, x 400).

DISCUSSION

Dox is a widely used chemotherapeutic agent in the treatment of several malignant solid tumours. However, multiorgan and particularly liver toxicity linked to Dox-treatment constitutes a serious draw back to its clinical use. It is commonly admitted that Dox-induced hepatotoxicity is linked to an oxidative stress status, characterized by reactive oxygen species (ROS) generation and disturbances in free iron and calcium levels. Controlling the redox balance as well as maintaining Ca^{2+} homeostasis could be a successful approach in the prevention of Dox toxicity.

In the present work, we showed that treatment of healthy rat with a single high dosage Dox (20mg/kg *bw*) induced an increase in liver lipoperoxidation but not carbonylation, a decrease in CAT activity and surprisingly an increase in POD and SOD activities along with a decrease in H_2O_2 and free iron but an increase in Ca^{2+}. Dox-treatment also increased plasma transaminases but with no effect on LDH activity.

Overall our data are consistent with several previous studies dealing with Dox-induced hepatotoxicity (for review see Cao *et al.*, 2012), mainly mediated by an oxidative stress status with some discrepancies however. We found that Dox did not affect protein carbonylation, which could be linked to the negative effect of Dox on free iron, as it was recently established that protein carbonylation is an important iron-dependant biological event (Wong *et al.*, 2012). Thus Dox-induced toxicity into the liver did not meet the "ROS and iron" hypothesis generally admitted for Dox-induced cardiotoxicity (Corna *et al.*, 2004). For instance Dox-induced hepatotoxicity is characterized by a decrease in free iron and H_2O_2, which is not in line with the decrease in CAT activity. However, Dox-induced increase in liver Ca^{2+} is in accordance with a plethora of animal studies dealing with Dox-induced Ca^{2+} overloading, rather than deficiency (Kalivendi *et al.*, 2005; Kalivendi *et al.*, 2001; Kim *et al.*, 2006). More generally, it is well established that intracellular Ca^{2+} homeostasis is linked to multidrug resistance in neoplastic cells (Sulová *et al.*, 2009) and the present experimental model is suitable to study the specific response of each peculiar organ following Dox-treatment. In this respect, regulation of multidrug resistance-associated protein 2 (Mrp2) by the type II inositol 1,4,5-triphosphate receptor (InsP(3)R2)-induced calcium signalling in mouse liver has been recently described (Cruz *et al.*, 2010) and L-type Ca^{2+} channel blockers as verapamil reverse antineoplastic drugs as vincristine-induced multidrug resistance in human lung cancer cell lines (Chiu *et al.*, 2010).

The most important result of the present study is the hepato-protection offered by GSSE against Dox-induced toxicity. Prevention and protection from Dox-induced

hepatotoxicity has been achieved with various agents including ω-3 fatty acids (Tulubas *et al.*, 2013), vitamin E (Shiva kumar *et al.*, 2012) and resveratrol (Oktem *et al.*, 2012). However the protection offered by a single antioxidant is limited. Multi-organ protection has been obtained using multiple anti-oxidant as chinese herbal medicine ANTIOXIN (Qin *et al.*, 2008) or a mixture of proanthocyanidins derived from grape seeds (Bagchi *et al.*, 2002). In our present case, GSSE offered efficient protection to the liver at high and safe dose of 500mg/kg, corresponding to 35g/day for a 70kg human adult. GSSE has even been used at very high dosage corresponding to 280g/day in 3 month- long experiments with no sign of toxicity nor adverse effects (unpublished data). In addition we found GSSE highly protective against Dox-induced morphological alterations of the liver, a property likely linked to calcium overloading (Simunek *et al.*, 2009). Interestingly, we recently demonstrated the protective effect of GSSE against high fat diet-induced heart (Charradi *et al.*, 2011), brain (Charradi *et al.*, 2012) and kidney (Charradi *et al.*, 2013) dysfunctions as well as on Dox-induced cardio toxicity (Mokni *et al.*, 2012). Moreover, proanthocyanidins from grape seeds were shown to enhance anti-tumor effect of Dox both *in vitro* and *in vivo* (Zhang *et al.*, 2005) without exerting any inverse dose-response effect as recently described for piquia pulp (Almeida *et al.*, 2012).

We did not know at the present time which polyphenol among those found in GSSE is responsible for such protection. To our opinion, the hepato-protection of GSSE described in the present study is unlikely linked to the specific effect of a single compound or polyphenol, but seems rather as the result of a synergism between several GSSE-containing compounds. In this respect, silymarin which is a complex mixture of flavonols and flavonoids, was shown to exert potent protection against Dox-induced liver toxicity and even potentiated Dox activity by inhibiting the drug efflux from cancer cells (Raskovic *et al* 2011). Similarly fenugreek seeds were recently shown to provide a good protection against Dox-induced oxidative stress and hepato-toxicity in rats (Sakr and Abo-Al-Yazid, 2012).

CONCLUSION

Our data showed that Dox-induced hepatotoxicity is partly mediated by oxidative stress and that GSSE, even at high dose is safe and liver protective and should be used as a co-chemotherapy to Dox use.

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