Hibiscus sabdariffa extract alleviate nephrotoxicity induced by adriamycin in male rats

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Adriamycin (ADR) is an effective anthracycline chemotherapeutic agent clinically used for treatment of several types of haematological malignancy and solid tumour. However, the risk of cardiac, renal, pulmonary, testicular, and hematological toxicities largely limits its effective and widespread use in clinical oncology. Hibiscus sabdariffa L. has shown to possess powerful antioxidant properties. This study aimed to investigate the impact of Hibiscus sabdariffa extract (HSE) on ADR-induced nephrotoxicity in male rats. There were four study groups i.e. control, ADR (10 mg/kg), and HSE+ADR groups (500 and 750 mg/kg), where rats received HSE for 1 week before ADR injection. After 14 days from ADR injection, serum was separation for determination of blood urea nitrogen (BUN) and creatinine. Renal tissues were subjected to determination of malondialdehyde (MDA) and glutathione peroxidase (GPx) contents, as well as examined microscopically. Results revealed that ADR induced significantly increased BUN, creatinine concentrations and renal content of MDA, with significantly decreased renal GPx levels as compared with control rats. Pretreatment of ADR-treated rats with HSE resulted in a significant improvement in tested kidney functions, as well as normalize renal contents of MDA and GPx. Histopathological examination of kidney sections revealed that, ADR caused many renal injury, while HSE pretreatment showed either slight congestion of glomerular tuft or no histopathological change at the high dose of HSE. These results suggested that, HSE is effective in ameliorated ADR-induced nephrotoxicity in male rats, thus may represent a promising new protective strategy against chemotherapy drugs. Therefore, it could be used in therapy and management of nephrotoxicity due to its antioxidant properties.

Key words: Adriamycin, Nephrotoxicity, Hibiscus sabdariffa, Antioxidant, Rats.
chemotherapeutic drugs for cancer treatment, but its use is limited by chronic and acute toxic side effects (Carvalho et al. 2009). Adriamycin-induced nephrotoxicity is a major cause of acute kidney injury (Dolin and Himmelfarb, 2008). The exact mechanism of ADR-induced nephrotoxicity is not yet completely understood. Nephrotoxicity induced by ADR may be part of a multi-organs damage mediated mainly through free radicals formation eventually leading to membrane lipid peroxidation (El-Sheikh et al. 2012).

Drugs can induce oxidative stress by generating free radicals that are mostly available as by-products or as aerobic metabolic products. These free radicals when generated excessively at cellular level may cause damage to tissue proteins, nucleic acids, and membrane lipids (Wang et al. 2000). Reactive oxygen species (ROS) are efficiently detoxified by antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in normal healthy conditions. However, under stress condition which causes excessive production of ROS, there is an imbalance between the production of oxidants and the defense systems of the organism which may promote the induction of lipid peroxidation, proteins and DNA damage, leading to cell death via apoptosis or necrosis (Klaunig et al. 2010; Goyal et al. 2016).

Hibiscus sabdariffa (HS) is a tropical annual herbal shrub which has been evaluated for treatment of various diseases like tumors, inflammation, heart diseases, diabetes, hyperlipidemia, and diarrhea (Rajesh et al. 2011; Kandhare et al. 2012). Its flowers contain many active constituents, mainly, cyanidin, quercetin, calcium oxalate, thiamine, riboflavin, niacin and ascorbic acid (Sikarwar and Patil, 2011; Ruban and Gajalakshmi, 2012; Muhammad and Shinkafi, 2014).

The most important bioactive constituent of HSE is anthocyanin that has been found to counteract the oxidative cellular damage during many diseases and toxicity conditions (Ali et al. 2005; Da-Costa-Rocha et al. 2014). By understanding the free radicals mechanism of anthracycline-induced nephrotoxicity, the hypotheses of this study is to investigate the possible protective role of HS extract (HSE) on ADR-induced renal damage in male albino rats.

MATERIALS AND METHODS

Plant material

Hibiscus sabdariffa L. extract, in tablet form was obtained from SWANSON Health Products Company. Each capsule contained (400 mg) of full spectrum HS flower, with purity (99%), there is up to a milligram of microcrystalline cellulose.

Drug, kits and chemicals

Adriamycin (Doxorubicin hydrochloride), red color powder, was obtained from King Abdulaziz University hospital. Blood urea nitrogen (BUN) (product number 1200801) and creatinine (product number 105102) Elisa kits were purchased from Cayman Chemical Company USA. Glutathione peroxidase (product number ab102530), and lipid peroxidation (MDA) (product number ab118970) Elisa kits were purchased from Abcamplc Global Medical Supplies. All chemicals were with the highest laboratory purity were purchased from Sigma Chemical Co., (St. Louis, MO, USA).

Animals

Male Wister albino rats (n= 24) (180-200 g) were obtained from the Animal experimental unit of King Fahd Center for Medical Research, King Abdullah University. All animals were allowed to one week acclimatize in animal housing standard laboratory conditions before being used for this study. They fed standard nutritionally balanced diet according to Reeves (1997), and drinking water ad libitum.

Pretreatment with Hibiscus sabdariffa

Hibiscus sabdariffa extract was prepared by dissolving HS powder in distilled water at the room temperature. Two oral doses of HS 500 and 750 (mg/kg b.w) were administrated by gavage to rats, the dose 500 mg/kg was chosen according to Kunworarah et al. (2014).

Experimental design

After one week for adaptation, rats were classified into four groups (6 rats each): Group (I): control rats received daily oral dose of dist. water (1ml) and intraperitoneal (i.p) injected with phosphate buffer saline (PBS).

Group (II) control positive group (ADR) rats received daily oral dose of dist. water (1ml), and i.p injected with a single dose of ADR (10 mg/kg b.w).

Groups (III and IV) ADR+ Pretreated groups with HSE; rats received orally HSE 500 and 750 (mg/kg b.w) dissolved in (1ml) of distal water for 7
days before and daily after ADR i.p. injection for 14 days.

Blood collection and serum separation
One day after the end of the experiment, after 21 days from the beginning of the study, blood samples were withdrawn by heparinized capillary tube from the retro orbital plexus of each rat under anesthesia with diethyl ether, then centrifuged at 3000 rpm for 15 min., to separate serum, which stored at -20°C for the biochemical analysis (Field et al. 1993). Kidney specimens collected and kept either frozen or in buffered 10% formalin solution.

Biochemical analysis
Determination of renal functions and renal oxidative stress. Serum blood urea nitrogen (BUN) and creatinine were measured according to Tietz (1986) and Husain et al. (2004), respectively. Renal thiobarbituric acid reactive substances (TBARS) were used to assay malondialdehyde (MDA) according to Ohkawa et al. (1979). The MDA-TBA adducts formed by the reaction of MDA and TBA under light temperature and acidic conditions is measured calorimetrically at 540nm. Renal glutathione peroxidase (GPx) activity was measured according to Rotruck et al. (1973), GPx reduces cumenehydroperoxide (CuOOH), while oxidizing GSH to GSSG. The generated GSSG is reduced to GSH with consumption of NADPH by GR. The decrease of NADPH measured at 340 nm is proportional to GPx activity.

Histological examination
Kidney from each group was removed, washed immediately with saline and then fixed in 10% buffered formalin for 24 hrs. Then embedded in paraffin, sections cut at 5μm and stained with Hematoxylin and Eosin (Bancroft et al. 1998). These sections were then examined under a light microscope for histological changes.

Statistical analysis
Statistical analyses of data were carried out using SPSS version 22. Data were expressed as the mean ± SEM. Comparison between groups will be done by one-way analysis of variance (ANOVA), followed by L.S.D (Snedecor and Cochran, 1989).

RESULTS
Renal functions enzymes
Treatment of rats with ADR (10 mg/kg) significantly increased both serum BUN and creatinine concentrations compared to control rats serum concentrations. Pretreatment of ADR treated rats with HSE (500 mg/kg) resulted in a non-significant decrease in both serum BUN and creatinine concentrations compared to ADR-treated rats serum concentrations. On the other hand, pretreatment of ADR treated rats with HSE (750 mg/kg) significantly decreased both serum BUN and creatinine concentrations compared to ADR-treated rats serum concentrations. Finally, pretreatment of ADR treated rats with HSE (750 mg/kg) significantly decreased both serum BUN and creatinine concentrations compared to HSE (500 mg/kg) treated rats serum concentrations, their values returned to the control rats serum concentrations (Table 1).

Oxidative stress in renal tissues
Treatment of rats with ADR (10 mg/kg) significantly increased kidney MDA content and significantly decreased kidney GPx enzyme activity compared to control rats value. On the other hand, pretreatment of ADR treated rats with HSE (750 mg/kg), significantly decreased kidney MDA content and increased kidney GPx enzyme activity compared to ADR-treatment rats values, their values returned to the control rats values. Finally, pretreatment of ADR treated rats with HSE (750 mg/kg) significantly decreased kidney MDA content and increased kidney GPx enzyme activity compared to HSE (500 mg/kg) treated rats values (Figures 1 and 2).

Histological examination
Sections from control rats, showing normal histological structure of renal parenchyma (Fig. 3-1). Sections from rats treated with ADR (10 mg/kg) showed vacuolation of epithelial lining renal tubules, congestion of glomerular tuft, hypertrophy, perivascular edema, presence of protein droplets in Bowman’s space, cystic dilatation of renal tubules and eosinophilic material in the lumen of renal tubules (Fig. 3-2.a-d). Sections from ADR rats pretreated with HSE (500 mg/kg b.w) showed congestion of glomerular tuft and renal blood vessels, and slight vacuolation of epithelial lining renal tubules and endothelial lining glomerular tuft (Fig. 3-3 a-b). Sections from ADR rats pretreated with HSE (750 mg/kg) showed no histopathological changes (Fig. 3-4).

DISCUSSION
Drug-induced nephrotoxicity is a major cause of acute kidney injury (Dolin and Himmelfarb, 2008). Nephrotoxicity is one of the most common adverse effects of ADR (Park et al. 2012).
Table 1: Effect of HSE on serum BUN and creatinine concentrations measured in ADR-induced nephrotoxicity in rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>ADR</th>
<th>ADR +HSE (500 mg/kg)</th>
<th>ADR +HSE (750 mg/kg)</th>
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<tr>
<td>BUN (mg/dl)</td>
<td>63.0 ± 0.67</td>
<td>183.0 ± 32.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>164.0 ± 17.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>89.0 ± 12.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.67 ± 0.04</td>
<td>1.82 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.69 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.89 ± 0.15&lt;sup&gt;c&lt;/sup&gt;</td>
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ADR: Adriamycin, HSE: Hibiscus sabdariffa L. extract, BUN: Blood urea nitrogen
Data are presented as mean ± SEM (n=6), <sup>a</sup> Significant versus control group, <sup>b</sup> Significant versus ADR group, <sup>c</sup> Significant between ADR + HSE (500 mg/kg) and ADR+ HSE (750 mg/ kg) groups, (<sup>*</sup>p ≤ 0.05, <sup>#</sup>p ≤ 0.01 and <sup>+</sup>p ≤ 0.001).

Although the exact mechanism of ADR-induced nephrotoxicity remains unknown. The disturbance in oxidant-antioxidant systems results in tissue injury that is demonstrated with protein oxidation in renal tissue is recognized as one of the possible biochemical mechanisms of ADR-induced nephrotoxicity (Karaman et al. 2006). In addition, ADR may have a direct renal damaging effect as it accumulates preferentially in the kidney. Furthermore, ADR-induced heart and liver toxicity may result in modulation of blood supply to the kidney and hence alter xenobiotic detoxification processes, thus indirectly contributing to ADR-induced nephropathy (Injac et al. 2008).

*Hibiscus sabdariffa* (HS) flowers comprises of many bioactive flavonoids compounds as anthocyanins, quercetin, cyanidin, kaempferol, hydrocitrlic acid, saponins, tannins, hemidesmine, hemidesmol, hemidesterol, stearoptin, pregnane glycosides, β-sitosterol, indicusin, coumarin, volatile oils and triterpines (Anoop and Jegadeesan, 2003; Akim et al. 2011). It has been used traditionally for the treatment of blood disorders (Anjaria et al. 2002), hypoglycemic (Murshead et al. 2005), antioxidant, antiinflammatory (Mary et al. 2003), anti-inflammatory (Lampronti et al. 2008), antiulcerogenic (Anoop and Jegadeesan, 2003), hepatoprotective (Obi and Uneh, 2003), cardioprotective (Gauthaman et al. 2006), neuroprotective (Nade et al. 2009), renoprotective (Kotnis et al. 2004), hypocholesterolemic and antihypertensive properties (Mckay et al. 2010). It is also known to have diuretic properties (Alarcón-Alonsoa et al. 2012). Numerous studies in vitro experiments have evaluated the effects of HS extracts against various cancer cell lines.
Figure 2: Effect of HSE on the renal tissue GPx (nmol/min/mg) activity in ADR-induced nephrotoxicity in rats.

ADR: Adriamycin, HSE: Hibiscus sabdariffa L. extract, GPx: Glutathione peroxidase
Data are presented as mean ± SEM (n=6), a significant versus control group, b Significant versus ADR group, c Significant between ADR + HSE (500 mg/kg) and ADR+ HSE (750 mg/ kg) groups. ( p ≤ 0.05, "p ≤ 0.01 and "p ≤ 0.001).

Proposed mechanisms of action focus on antioxidant activity and the ability to induce apoptosis of cell line (Wang et al. 2003; Mei et al. 2005). Therefore, the hypotheses of this study are to investigate the possible protective role of HS extract (HSE) on ADR-induced renal damage in male albino rats.

Effect of HSE on ADR-induced changes in kidney functions
The results of the present study revealed that, treatment of rats with ADR (10 mg/kg) significantly increased both serum BUN and creatinine concentrations compared to control rats. Similar results are in agreement with those obtained by Al-Saedi et al. (2014); Su et al. (2015) and Refaie et al. (2016), who reported that, injection of rats with ADR resulted in a significant increase in serum creatinine and urea levels compared to control group. Also our study findings were in agreement with many other previous studies results which find increased serum concentrations of urea and creatinine after ADR injection as markers of nephrotoxicity (El-Sheikh et al. 2012; Koul et al. 2013).

Urea and serum creatinine are the most sensitive markers of nephrotoxicity implicated in the diagnosis of renal injury (Khan and Sultana, 2004). The nephrotoxic effect of ADR is characterized by decreasing glomerular filtration rate leading to a rise in serum urea and creatinine (Refaie et al. 2016). The elevation of serum urea and creatinine is observed after renal damage (Ferguson et al. 2008). Previous study of ADR induced nephrotoxicity concluded that, administration of ADR changes the kidney enzymes which can be attributed to ADR induced inflammation (Zordoky et al. 2011). Where, ADR-induced inflammatory response of the kidney tissue exhibited leukocytes accumulation in the renal tissue that led to production of hypochlorous acid which responsible for damaging and oxidizing proteins, amino acids, nucleic acids and lipids of kidney tissues (Arnhold et al. 2001). Furthermore, ADR-induced inflammation which is manifested as neutrophils infiltration may enhanced ROS production and hence nephrotoxicity (Deman et al. 2001).

Pretreatment of ADR treated rats with HSE (750 mg/kg) significantly decreased both serum urea and creatinine concentrations compared to ADR-treated rats, moreover returned both serum urea and creatinine concentrations to the control rats serum concentrations. In agreement with our results Ademiluyi et al. (2013) reported that, administration HS calyx red dye for 20 days
Control rats showing normal histological structure of renal parenchyma (1). Rats injected with ADR showing vacuolation of epithelial lining renal tubules and congestion of glomerular tuft (2.a), hypertrophy and vacuolation of glomerular tuft and perivascular edema (2.d), hypertrophy, congestion and vacuolation of glomerular tuft as well as presence of protein droplets in Bowman's space (2.c) and cystic dilatation of renal tubules and eosinophilic material in the lumen of renal tubules (2.d). ADR rats pretreated with HSE (500 mg/kg) showed congestion of glomerular tuft and renal blood vessels (3.a), and slight vacuolation of epithelial lining renal tubules and endothelial lining glomerular tuft (3.b). ADR rats pretreated with HSE (750 mg/kg b.w) showed no histopathological changes (4). (H&E x 400)

resulted in reduction in the serum creatinine and urea levels in cisplatin-induced nephrotoxicity. Ibrahim and Albadani (2014) reported that, administration of HSE before gentamicin resulted in decreased both BUN and serum creatinine. Kunworarath et al. (2014) and Okoko and Oruambo (2008) reported that, HSE administration improved serum BUN and creatinine in cisplatin-induced nephrotoxicity study. In addition, Ademiluyi et al. (2013) reported that, sorghum (Sorghum bicolor) straw dye which is rich in proanthocyanidins administration reversed the plasma creatinine, urea, and blood urea nitrogen near to the normal levels in cisplatin-induced nephrotoxicity and antioxidant status in rats. The improvement in BUN after HSE administration could be attributed to HSE high content of several antioxidants including anthocyanine, flavonoids and phenolic acids (Liu et al. 2002; Farombi and Fakoya, 2005).
Effect of HSE on ADR-induced changes in kidney oxidative stress

This study results revealed that, treatment of rats with ADR (10 mg/kg) significantly increased kidney MDA content with significantly decreased kidney GPx enzyme activity compared to control rats value. The present results are in agreement with those obtained by Refaie et al. (2016), who reported that, injection of rats with ADR resulted in increased renal MDA. Also, our study findings were in agreement with many other previous studies results which find increased lipid peroxidation in the renal tissue and decrease in the levels of GPx after ADR injection as markers of oxidative stress (El-Sheikh et al. 2012; Koul et al. 2013; Al-Saedi et al. 2014 and Su et al. 2015). Oxidative stress may damage cellular structures via lipid peroxidation of cellular membranes. Superoxide anion reacts with lipid to form lipid peroxides followed by β-oxidation to form MDA (Morsy et al. 2013). Adriamycin-induced increase in the kidney MDA content suggests oxidative stress which is the result of increased ROS production as well as depletion of kidney antioxidant enzymes (Ademiluyi et al. 2013).

Pretreatment of ADR treated rats with HSE (750 mg/kg) significantly decreased kidney MDA content and increased kidney GPx enzyme activity compared to ADR-treatment rats values, their values returned near to the control rats values. Similar findings were found by Ademiluyi et al. (2013) and Kunworarath et al. (2014), who reported that, HSE administration return MDA level back to control value in cisplatin-induced nephrotoxicity study. Furthermore, Lakshmi et al. (2014) reported that, black grapes proanthocyanidin enriched extracts administration increased GPx activity enzymes and decreased MDA levels in ADR-induced nephrotoxicity study. One of the likely mechanisms in improving this renal damage may be due to the antioxidative property of HSE as discussed by Kunworarath et al. (2014). They concluded that, the acute treatment with HSE in cisplatin treated rat attenuate the elevation of MDA level confirming its possible roles as an antioxidative substance. The chemical constituents of HSE responsible for the reduction of renal MDA level in cisplatin treated rats are likely to be quercetin and/or delphinidine 3-sambubioside (anthocyanin compounds) which have been reported to possess antioxidative properties (Cao et al. 1997; Tsai et al. 2002). Findings have shown that dried calyx of HS is rich in various phytochemicals such as anthocyanins, tannins, phenolic acids, phytoesters and policosanols (Carvajal-Zarrabal et al. 2012). In addition, calyces of HS contain potent antioxidant components including vitamin C and tocopherol; this explains the protective effect of this plant.

Anthocyanins have been shown to possess several therapeutic properties such as, hepatoprotective, anti-inflammatory, anticancer and chemoprotective (Zhang et al. 2011; Carvajal-Zarrabal et al. 2012). Ademiluyi et al. (2013) study determined anthocyanin content of the HSE calyx dye to be 121.5 mg cyanidin-3-rutinoside equivalent/100 g, thus, the observed nephroprotective effect of HS dye could be attributed to its anthocyanin content. Anthocyanins are potent antioxidants, capable of inhibiting lipid peroxidation and scavenging reactive oxygen species such as hydroxyl free radical (Ologundudu et al. 2010). Previous studies have demonstrated the antioxidant and drug detoxification potentials of HS calyx anthocyanins extracts (Ajiboye et al. 2011; Ademiluyi et al. 2013).

Effect of HSE on ADR-induced kidney histopathological changes

The present results showed that injection of rats with ADR (10 mg/kg) resulted in many histopathological changes in rat’s kidneys tissue. Two weeks after ADR injection, the kidneys sections showed either vacuolation of epithelial lining renal tubules, eosinophilic materials, crystals in the lumen of renal tubules or cystic dilatation of renal tubules. As well as, perivascular oedema, as well as protein droplets in Bowman’s space. Similar results were obtained by Refaie et al. (2016), who reported that, scarified rats after 4 days of injection with ADR (15 mg/kg) showed marked enlargement of some vascular glomeruli which tightly fill the renal corpuscles. Most renal corpuscular and tubular cells showed abundant cytoplasmic vacuolations and tubular distortion. Interstitial inflammatory cells infiltrations were also observed. Coincide with our study findings of Al-Saedi et al. (2014); Su et al. (2015) and Mohan et al. (2010).

Sections from ADR rats pretreated with HSE (750 mg/kg) showed no histopathological changes. In agreement with this study results, Mokni et al. (2016) showed a potential protective effect of grape seed and skin extract which is rich in phenolics and flavonoids against ADR-induced nephrotoxicity and histopathological changes. Furthermore, Nirwane et al. (2014) showed a potential protective effect of Punica granatum seeds which is rich in anthocyanidins against ADR-induced nephrotoxicity and histopathological
changes. This may be explained by the chemical constituents of HSE (anthocyanin compounds) which have been reported to possess antioxidative properties.

CONCLUSION
The present findings demonstrate that HS has multiple therapeutic activities that are beneficial in the kidney and thus HS is a promising candidate to prevent and block ADR nephrosis. Further studies should be conducted for longer period and with different doses of HS.

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