Physiology and Pharmacology

Physiol Pharmacol 22 (2018) 269-278 **www.phypha.ir/ppj**

Original Article

Effect of *Plantago major* extract on doxorubicin-induced nephropathy in rat

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Abstract

Introduction: Nephropathy is defined as rational loss of renal function related with glomerulosclerosis and declining glomerular filtration rate. Inflammation and oxidative stress play a critical role in nephropathy. *Plantago major* has antioxidant effects. The aim of present study is the investigation of the effect of *Plantago major* hydro-alcoholic extract on the oxidative stress and renal function in kidney of rat.

Methods: Rats were divided into five groups: control (Co), doxorubicin (DOX), doxorubicin+vitamin E (DOX+Vit E), 600mg/kg *Plantago major* (PM)+doxorubicin (PM600+DOX), 1200mg/kg *Plantago major* (PM)+doxorubicin (PM1200+DOX). DOX (5mg/kg, IV), Vit E and PM extract (600 and 1200mg/kg, PO) were administrated for 35 days. Finally, urine, blood samples and renal tissue were collected to measurement of redox markers, functional parameters and renal index percentage.

Results: The renal superoxide dismutase (SOD) activity, total thiol and functional parameters significantly reduced and malondialdehyde (MDA) concentration increased in DOX group in comparison with control group. The renal SOD, catalase activities and total thiol content were significantly increased and MDA level decreased in PM treated groups along with DOX group in comparison with DOX group. The functional parameters significantly enhanced in treated groups with PM in comparison with the DOX group. The extract did not relive enhanced % renal index induced by DOX.

Conclusion: Hydro-alcoholic extracts of PM, specially at its high dose led to an improvement in DOX-induced renal function and oxidative stress.

Keywords:
Doxorubicin;
Plantago major;
Oxidative stress;
Renal function

Received: 7 Apr 2018
Accepted: 15 Oct 2018

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Introduction

Nephropathy is defined as rational loss of renal function related with glomerulosclerosis, declining glomerular filtration rate (GFR), nephrotic syndrome, enhanced arterial blood pressure and fluid retention (Mohebbati et al., 2016a). Two major factors that

contribute to nephropathy are excessive oxidative stress and inflammatory responses (Yao et al., 2017). The levels of reactive oxygen species (ROS) increased in the nephropathy and levels of antioxidant agents reduce (Medina-Navarro et al., 2014).

There is several models for induction of nephrosis by some drugs such as gentamycin (Baradaran et al., 2014; Derakhshanfar et al., 2007), cisplatin (Ashrafi et al., 2012) and vancomycin (Karami et al., 2018). Doxorubicin (DOX) with commercial name of adriamycin, an anthracycline antibiotic, is used for the treatment of many cancers and diseases such as human neoplasm, leukemia, solid tumors, breast, lung, gastric, ovarian, thyroid and others (Oguz et al., 2016). The use of DOX is limited due to its adverse side-effects such as acute nausea and vomiting, baldness, gastrointestinal problems and disturbances to the neurological system (Tseng, 2016). Also DOX induces serious toxicity in the heart and kidney (Tacar et al., 2013), as it leads extensive nephrotoxicity (Mahmoud, 2016) and cardiotoxicity (Jovanović et al., 1995). DOX-induced changes in the kidney of rats include increases in glomerular capillary permeability and glomerular atrophy (Injac et al., 2008). In animal experiments, DOX has showed the nephrotoxic activity and induces the chronic progressive glomerular disease that results in nephropathy and heavy proteinuria (Injac et al., 2008; Kumral et al., 2015; Mohebbati et al., 2017). Although the clear mechanism of nephrotoxicity induced by DOX remains unknown, currently the belief is spread that free radical generation is importantly involved in the cytotoxicity mechanism induced by DOX (Mohebbati et al., 2016b; Mohebbati et al., 2017). Inside the cell, ROS may attack and injure multiple molecules including DNA, lipids as well as proteins and also activate some important signaling pathways that lead to necrosis and apoptosis (Parhizgar et al., 2016). Therefore, Oxidative stress is considered as leading cause of renal failure in nephrotoxicity (Song et al., 2018; Mohebbati et al., 2016c).

Plantago major (PM) is an herbal plant that belongs to the Plantagiaceae family. The PM is a plant with wide geographic distribution in temperate grassland of the world. It naturally grows in Central Asia and Northern Europe but now it is almost found all over the world (Haddadian et al., 2014). This plant has been showed to contain 5 groups of biochemically active compounds including benzoic compound, flavonoids such as baicalein, luteolin, baicalin, iridoid glycoside, phenolic compounds such as caffeic acid, chlorogenic acid, ferulic acid, p-coumaric acid and triterpenes (Duke, 1992). In traditional medicine, PM was used as astringent (Parhizgar et al., 2016), anesthetic (Núñez Guillén et al., 1997), anti-inflammatory (Flores et al., 2016), antitumor (Oto et al., 2011), analgesic (Núñez Guillén et al., 1997), antiviral (Chiang et al., 2002), analeptic (Ozaslan et al., 2007) and anti-ulcer (Samuelsen et al., 1996). Nowadays this is commonly used in the treatment of a number of diseases associated to the digestive, respiratory, circulation and reproduction organs as well as cancer and infections (Chiang et al., 2002). The aim of present study was to investigate the possible protective effects of PM on DOX-induced nephrotoxicity in the rat kidney.

Materials and methods

Extract preparation

The PM was purchased from herbal store in Mashhad, Khorasan province, Iran. About 100g of powder of all dried parts of the plant, including the leaves, roots, stem and seed were homogenized in 1 liter of 70% ethanol and left to soak for 72h at 37°C with shaking. Next, the final mixture was filtered and the resulting liquid was concentrated under reduced pressure at 45°C in an EYELA rotary evaporator. Finally, the concentrated extract was kept in the incubator at 45°C for 72h to evaporate the ethanol residue yielding the crude extract (Salama et al., 2013).

Chemicals and drugs

The chemical materials were purchased from Merck company (Germany) and DOX was purchased from EBO pharma, Tehran, Iran.

Animals and treatment

Forty male Wistar rats (240±10g) were kept in an animal lab with standard condition. The rats were allowed to have access to water and food freely. Ethical code is IR.MUMS.fm.REC.1396.470. Animals were randomly divided to five groups (n=8 in each group) including: control (Co), doxorubicin (DOX, 5mg/kg) (Mohebbati et al., 2016c), vitamin E (100mg/kg) plus DOX (Vit E+DOX) (Shaikh et al.,

1999), PM at doses of 600mg/kg plus DOX (PM600 + DOX) (Parhizgar et al., 2016) and PM at doses of 1200 mg/kg plus DOX (PM1200 + DOX) (Parhizgar et al., 2016).

Sampling

Serum and urine samples were collected in days 0, 14, 21, 28 and 35 of the study. Blood collected from orbital sinus (1ml in each time) and urine collected using the metabolic cage. At the end of the experiment, the animals were euthanized by urethane. The renal tissue was removed, washed and after weighing, stored at -20°C. Weighing of all rats performed in the first and the end of the experiment period.

Malondialdeyde (MDA) and thiol assessment

The renal tissues were homogenized with cold KCl (150mM) for the measurement of thiol and MDA levels. MDA level is as a lipid peroxidation index. MDA reacts with thiobarbituric acid (TBA) as a TBA reactive substance (TBARS) and forms a red complex. One ml of homogenates was added to 2ml of a complex solution containing trichloroacetic acid (TCA)/TBA/hydrochloric acid and it was boiled in a benmarry for 40 minutes. After reaching to the lab temperature, the solution was centrifuged for 10 minutes at 1000g. Finally, the absorbance of the supernatant was measured at 532nm and tetraethoxypropane was used to prepare a standard curve at concentration ranges between 0.01-0.2 (µmol/l) (Janero, 1990). Absorbance at 532nm-absorbance at 600nm is absorbance due to MDA-TBA abduct. Extinction coefficient of this MDA-TBA abduct at 532 nm is 155 mM-1cm-1.

Concentration of MDA (mM)= (A532 - A600)/155. Let us presume: (i) A532 is 0.75; (ii) A600 is 0.05; (iii) volume of reaction mixture is 2 ml (one ml sample + one ml 0.5% TBA in 20% TCA) and (iv) path length is one cm, thus, concentration of MDA (mM)= (0.75-0.05)/155= 0.00387. Concentration of the MDA was calculated according to following equation: MDA concentration (M)= absorbance/(1.56 × 105 cm-1 M-1). The MDA concentration results are expressed micromole per gram of tissue.

DTNB (2, 2'-dinitro-5, 5'-dithiodibenzoic acid) reagent, that reacts with the -SH group, was used to measure total thiol content. The generated yellow complex has a peak absorbance at 412nm. The 50µl of renal tissue homogenates was added to 1ml Tris-EDTA buffer (pH=8.6) and the absorbance was read at 412nm versus Tris-EDTA buffer alone (A1). After that, 20µl of 10mM DTNB solution was mixed with the solution and it was kept in lab temperature for 15 minutes and the absorbance was read again (A2). The absorbance of DTNB reagent was read as blank (B) (Sharma et al., 2006). The thiol contents were determined by a spectrophotometric method based on the use of Ellman's reagent and the results are expressed as per gram of tissue: total thiol concentration (mM)= (A2–A1– B)× 1.07)/ 0.05× 14,150)

Measurement of superoxide dismutase (SOD) activity

SOD activity was determined by the procedure of Balasubramanian and Madesh (1998). This method is based on the colorimetry in which superoxide produced by auto-oxidation of pyrogallol and reduction of tetrazolium dye, MTT (3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide) to its formazan by SOD was done. It is determined at 570nm. One unit of SOD activity was defined as the amount of enzyme resulting 50% inhibition in the MTT reduction rate.

Determination of catalase (CAT) Activity

CAT activity of the kidney tissue was determined spectrophotometrically at 240nm in renal tissue homogenates using of the Aebi method with hydrogen peroxide (30mM) as the substrate (Cohen et al., 1970).

Biochemical assessment

Serum and urine samples were taken from all rats to determine serum creatinine, urea, albumin and urine creatinine and protein levels using albumin, urea and creatinine kits (Pars Azmoun Company, Tehran, Iran). Urine total protein determination by a trichloroacetic acid (TCA) precipitation method was automated on a Cobas Bio centrifugal analyzer. Then, glomerular filtration rate (GFR) estimated using creatinine clearance.

Data analysis

All data were expressed as mean±SEM. Normality test (Kolmogorov–Smirnov) was done. Different groups were compared by one and two way ANOVA

Table1: Comparison of the antioxidants and functional parameters between control groups of Plantago major. Data are presented as mean±SEM.

Parameters / Groups	GFR (ml/min)	Urea (mg/dl)	Urine protein excretion rate (mg/day)	Protein clearance (ml/day)	MDA (nmol/gr)	total thiol (µmol/gr)	SOD (U/mg)	Catalase (U/l)
Control	0.47±0.09	49±4.4	54±7.0	1.5±0.2	10.5±0.6	2.3±0.2	9.8±0.3	0.8±0.05
***Plantago major* (600mg/kg)**	0.46±0.10	55±6.0	59±9.0	1.5±0.6	9.3±0.9	2.5±0.3	10.1±0.9	0.8±0.08
***Plantago major* (1200mg/kg)**	0.46±0.06	52±4.6	56±10	1.6±0.3	9.0±0.8	2.9±0.5	10.2±0.8	0.9±0.09

Table 2: Comparison of GFR between different days in five groups. Data are presented as mean±SEM. **P*<0.05 and ***P*<0.01 compared to control group. $^{\#}$*P*<0.05 compared to DOX group.

Days GFR (ml/min)	Day0	Day14	Day21	Day28	Day35
Co	0.48±0.1	0.42±0.05	0.48±0.04	0.42±0.08	0.47±0.09
DOX	0.48±0.06	0.42±0.1	0.37±0.08	0.35±0.04*	0.27±0.04**
Vit E+DOX	0.45±0.07	0.49±0.04	0.37±0.04	0.37±0.05	0.28±0.03
PM600+DOX	0.48±0.08	0.51±0.05	0.5±0.09$^{\#}$	0.4±0.05	0.34±0.05$^{\#}$
PM1200+DOX	0.46±0.1	0.5±0.09	0.48±0.06$^{\#}$	0.46±0.09$^{\#}$	0.4±0.07$^{\#}$

Co:control, DOX: doxorubicin, Vit E: vitamin E and PM: Plantago major

followed by tukey's Post Hoc comparison test. Differences were considered statistically significant when *P*<0.05.

Results

In DOX group, the urine protein excretion rate and protein clearance significantly increased and GFR as well as antioxidant activity significantly decreased over the time. Comparison of the antioxidants as well as functional parameters in the control groups has been indicated in Table1. The results of the present study demonstrated that the MDA level was higher in DOX group in comparison with the CO group (*P*<0.001). On the other hand, the MDA concentration in DOX+Vit E, PM600+DOX and PM1200+DOX groups, significantly decreased compares to DOX group (*P*<0.01 and *P*<0.001; Fig. 1A).

Also, the total thiol content was lower in DOX group in comparison with the CO group (*P*<0.001). The total thiol content in DOX+Vit E and PM600+DOX groups, showed no significant increase in comparison with DOX group but in PM1200+DOX group compared to DOX group concentration of total thiol content significantly increased (*P*<0.01; Fig. 1B).

The results indicated that the SOD activity was reduced in DOX group compared to Co group (*P*<0.001). The SOD activity in DOX+VIT E, PM1200+DOX (*P*<0.001) and PM600+DOX (*P*<0.01) groups significantly increased compared to DOX group (Fig. 1C).

According to the present study, the catalase activity was lower in DOX group compared to Co group (*P*<0.001). The SOD activity in DOX+VITE, PM600+DOX (*P*<0.05) and PM1200+DOX (*P*<0.001) groups significantly increased in comparison with DOX group (Fig. 1D).

The results revealed that GFR levels in days 28 (*P*<0.05) and 35 (*P*<0.01) in DOX group significantly decreased compared to the control group. In treated rats with PM at the last days, the GFR significantly increased compared to the DOX group (Table2). The results indicated that serum urea in all days from each group was not significant (Fig. 2A).

The results showed that urine protein excretion rate as well as protein clearance at the last days in DOX group (*P*<0.05 to *P*<0.001), significantly increased compared to control group. The treated rats with PM

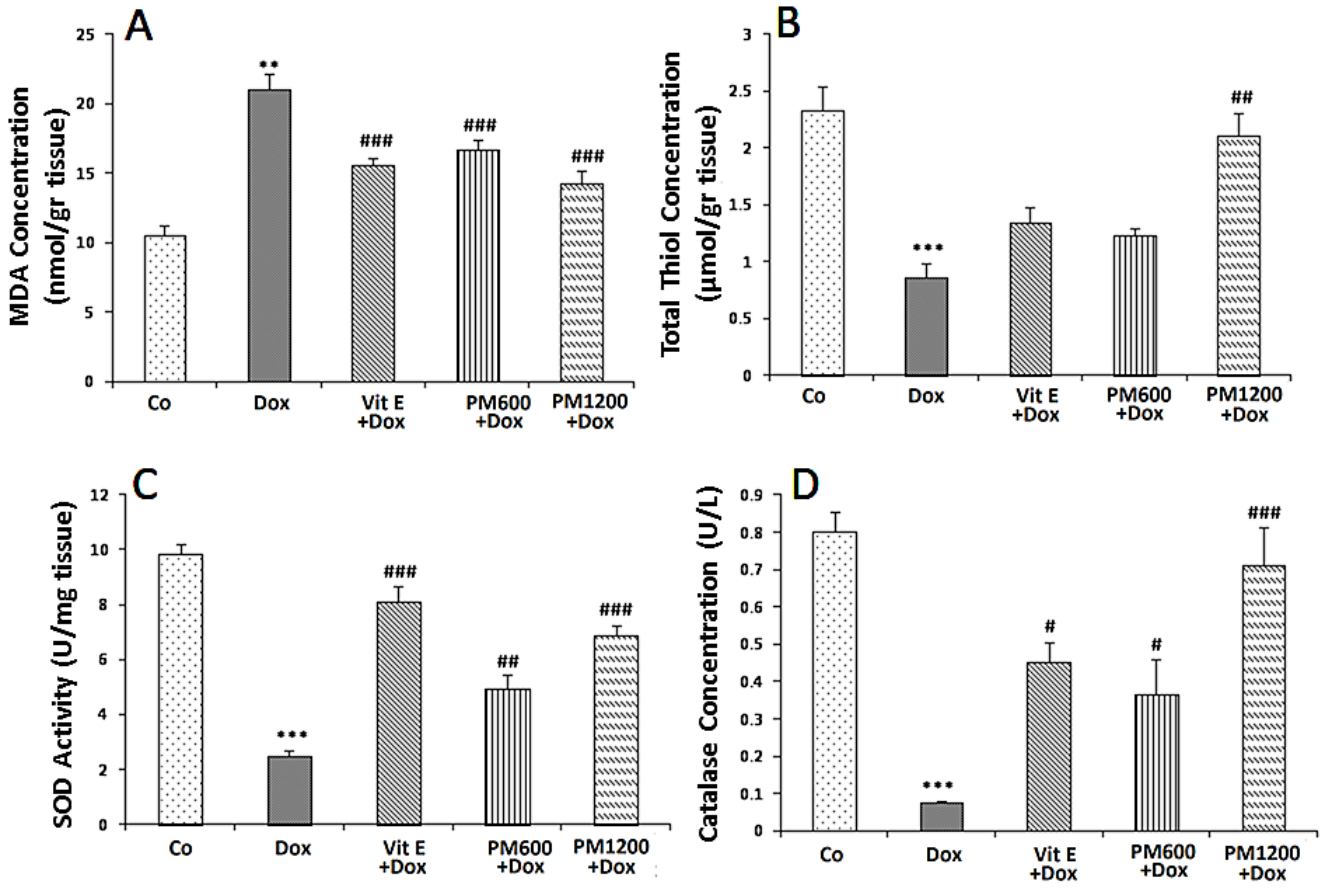

Fig.1. Comparison of the MDA (A) and total thiol (B) contents and SOD (C) and catalase (D) activities in renal tissue of five groups. Data are presented as mean±SEM (n= 8 in each group). ***P*<0.01 and ****P*<0.001 compared to control group. $^{\#}P$<0.05, $^{\#\#}P$<0.01 and $^{\#\#\#}P$<0.001 compared to DOX group. Co:control, DOX: doxorubicin, Vit E: vitamin E and PM: *Plantago major*

have been shown the significant decreased of the urine protein excretion rate at the last days compared to the DOX group (Figs. 2B and C).

The weight difference (between day 0 and 35) in DOX group in comparison with Co group significantly decreased (*P*<0.05; Fig. 3A). The renal index in DOX group in comparison with Co group significantly increased (*P*<0.01; Fig. 3B).

After checking the data normalization by Kolmogrov-Spirnov test, bivariat correlation test has been done. Generally, between oxidative stress agents and protein clearance (not GFR) correlation was calculated: (r=-0.6, *P*<0.01 for SOD, CAT, thiol/ protein clearance) and (r=0.6, *P*<0.001 for MDA/ protein clearance).

Discussion

The results of this study showed a reduction of oxidative stress factors (thiol, MDA, SOD and catalase) induced by doxorubicin in the groups treated with PM extract with doses 600 and 1200, respectively, comparable to vitamin E (an antioxidant agent), which indicates the beneficial effects of PM on the oxidative stress caused by doxorubicin.

In the renal system, doxorubicin causes an increase in the excretion of N-acetyl glucosamine, glycosaminoglycan and fibronectin from the urine, decreases the antioxidant enzymes activity such as glutathione and glutathione peroxidase, as well as increases the induction of microsomal and mitochondrial lipid peroxidation and hydrogen peroxidase (Bertani et al., 1982). Also, doxorubicin decreased the renal function by many mechanisms including GFR reduction (Mohebbati et al., 2017), instability of the glomerular basement membrane, and reducing the thickness of glycolic acid in the glomerular endothelium. One of the major side effects of this drug is proteinuria and renal toxicity. Also, the generation of ROS and free radicals play a role as the main mediators in this field (Egger et al., 2015). Our previous studies have shown that urea level in

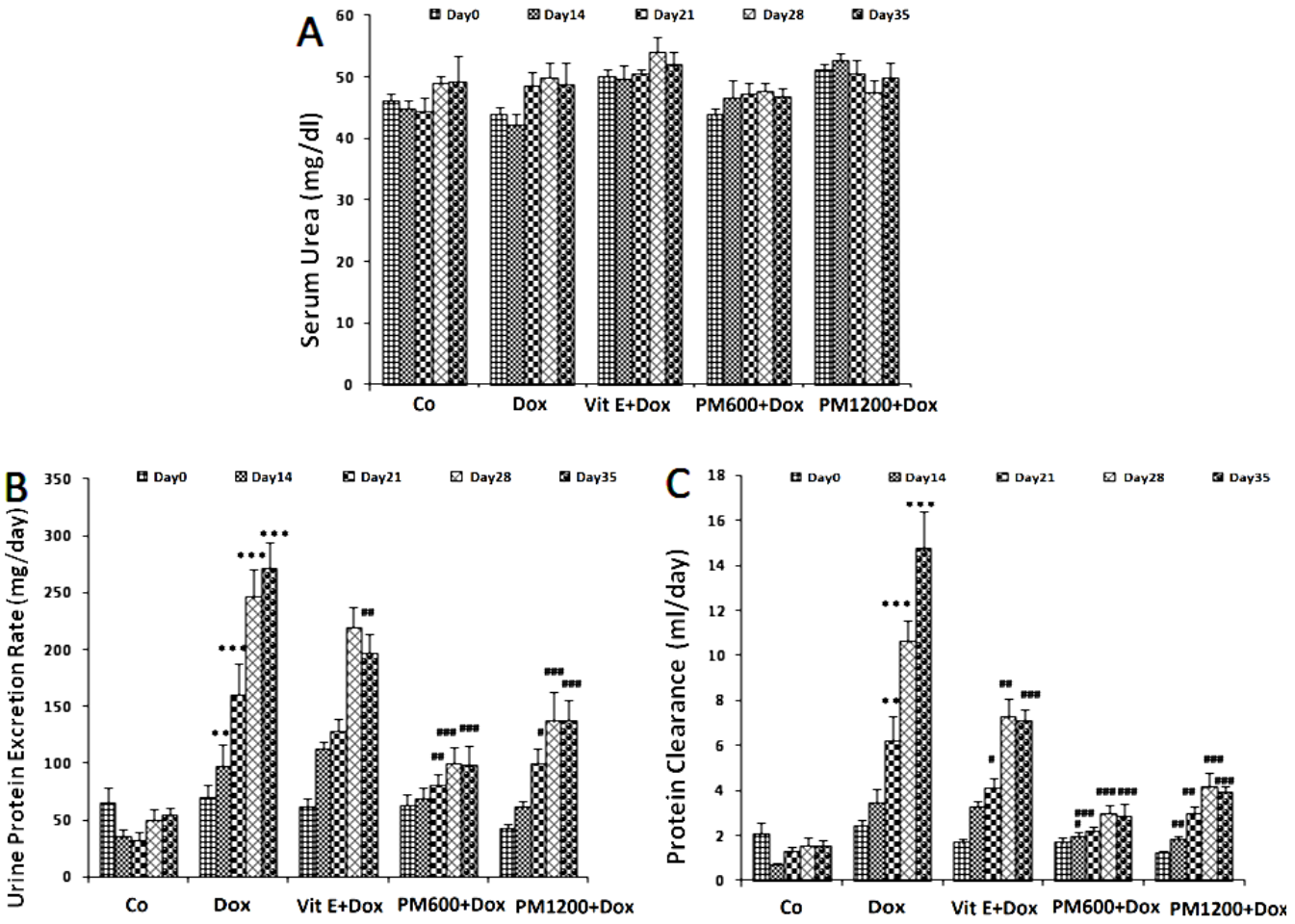

Fig.2. Comparison of serum urea (A), urine protein excretion rate (B) and protein clearance (C) between different days in five groups. Data are presented as mean±SEM. Co:control, DOX: doxorubicin, Vit E: vitamin E and PM: *Plantago major*

nephrosis induced by doxorubicin has not any changes. Therefore, the base of nephrosis induction in this study is reduced GFR as well as proteinuria (Mohebbati et al., 2017; Mohebbati et al., 2016c).

Oxidative stress plays a critical role in nephropathy caused by doxorubicin. Renal oxidative stress is caused by an increase in the formation of reactive molecular oxygen species, such as H_2O_2 and O_2, due to the reduction of the activity of antioxidant enzymes. Increasing the ROS generation increases the production of highly reactive free radicals such as hydroxyl, which reacts with cell components such as lipids, DNA and proteins, and ultimately causes loss of cell integrity and impaired function (Beheshti et al., 2017). The oxidative stress induces nephropathy by damaging the glomerular membrane through damage of endothelial cells, the pelvic cells or other components of the glomerular membrane (Nath and Norby, 2000).

In the present study, the MDA concentration of the kidney tissue in the doxorubicin group enhanced significantly compared to the control group. Also, the total concentration of thiol groups and activity of SOD and catalase enzymes in the kidney tissues of animals treated with doxorubicin showed a significant decrease compared to the control group.

Together with the findings from the present study, in a study done by Awwad in 2017, intravenous injection of 4mg/kg doxorubicin reduced the activity of antioxidant enzymes SOD and catalase in rat kidney tissue (Awwad et al., 2017). In a study conducted by Mohebbati in 2016, the preventive effect of *Nigella sativa* hydro alcoholic extract on oxidative stress induced by doxorubicin in rat kidney was evaluated. In this study, intravenous injection of doxorubicin with dose 5mg/kg resulted in an increase in the MDA and decreased activity of SOD and catalase enzymes and total thiol concentrations in kidney tissues. In treated groups, *Nigella sativa* leads to renal protection against doxorubicin. The considered plant has many

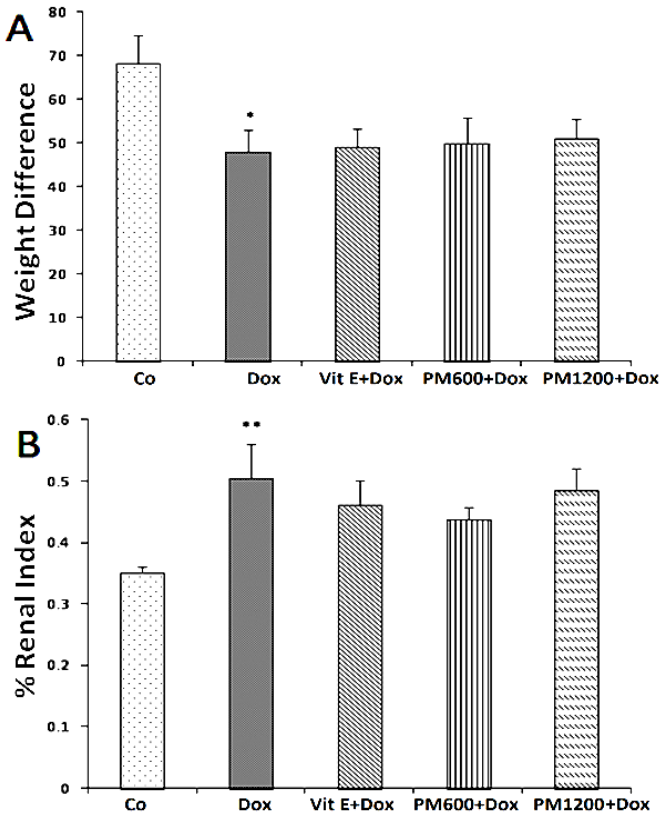

Fig.3. Comparison weight difference (A) and renal index (B) in five groups. Data are presented as mean±SEM. **P*<0.05 and ***P*<0.01 compared to control group.
Co:control, DOX: doxorubicin, Vit E: vitamin E and PM: *Plantago major*

antioxidant agents such as flavonoids and vitamins that result in its renal protection (Mohebbati et al., 2016a).

Doxorubicin lead to reductive activity in the cell. Bio reductive activity of doxorubicin is performed through interaction with several oxidoreductases, including cytochrome P450, NADPH-dependent reductase in the endoplasmic reticulum and nuclear coating, as well as NADH dehydrogenase in the mitochondrial electron transfer chain. In fact, oxidoreductases are capable of converting doxorubicin into a semiquinone radical through a capacity reduction mechanism. In fact, taking electrons from NADPH or NADH and its derivation with doxorubicin by oxidoreductases produces semiquinone radical (Davies and Doroshow, 1986).

This toxic radical, in aerobic conditions, can quickly react as an auto oxidase with oxygen as an electron receptor and produce ROS. Also, doxorubicin has a great tendency to mitochondrial membrane, thus providing a great deal of access to the electron transfer chains (Berthiaume and Wallace, 2007). The molecule that interacts with doxorubicin in the electron transfer chain is the complex I. By providing an electron to doxorubicin, this complex converts it into a free radical form capable of donating electrons to oxygen. Consequently, produces the ROS in the mitochondria. The result of the ROS production in pathogenesis is the change in the lipid, protein, nucleic acid, biological molecules and several signaling molecules (Sun et al., 2016).

Doxorubicin lead to DNA damage, it can be concluded that doxorubicin can reduce the expression of genes associated with antioxidant enzymes (Li et al., 2000). In a study by Parhizgar et al. (2016), the protective effect of PM on nephrotoxicity and oxidative stress caused by cisplatin was investigated. In this study, it was shown that PM in doses of 600 and 1200, could significantly reduce the oxidative stress parameters of cisplatin in rat kidneys. Our study also revealed this anti-oxidant effect of PM in rats treated with doxorubicin.

Studies have shown that the extract of PM with compounds such as phenolic acid, flavonoids, coumarin and lignans can have a sweeping effect on reactive species of oxygen and nitrogen (Beara et al.,

2012). Phenolic compounds in the PM prevent the destruction of DNA in the presence of free radicals. The caffeic acid derived from PM named plantamajuside, has an anti-inflammatory and antioxidant activity (Oto et al., 2011). The antioxidant activity of PM can be associated with the presence of phenylpropanoid glycoside and isomatarninoside (Kolak et al., 2011).

Polysaccharides extracted from PM seeds can destroy DPPH radicals and superoxide and hydroxyl radicals as well as inhibition of lipid peroxidation (Yin et al., 2010). PM extract with active biological substances such as flavonoids including apigenine, bicalein, bicalin, luteolin, hyspidoline, plantajinin, naptin and also the most important flavonoids, which can be mentioned as luteolin-7-OB-glucoside, have an effective role in decreasing of oxidative damage. Also, due to the direct relationship between inflammation and oxidative stress, one of the possible mechanisms of antioxidant effect of PM extract may be inhibition of inflammation (Zhou et al., 2013).

Current results suggest that dysfunction of the proliferator-activated receptor-γ coactivator (PGC)-1α-mitochondria axis is highly involved in podocyte injury induced by ADR (Zhu et al., 2014) and the previous studies confirm it (Egger et al., 2015; Jeansson et al., 2009). In this study, PM600 extract has an optimal effect on decreasing protein excretion rate and protein clearance. The proteinuria improvement was followed by the administration of DOX, which may be due to phenolic compounds, antioxidant properties and prevention of lipid peroxidation (Mohebbati et al., 2016b).

Probably, DOX with oxidative effects itself damages the endothelial cells of the vessels in the kidney and leaks proteins from the vessel to the interstitial fluid. Inflammation effects of DOX can also lead to inflammation and weight gain in the kidney and thereby increase kidney weight (You et al., 2011).

One of the most important limitations of this study was the lack of uniformity of biochemical parameters data on the first day of sampling, as well as the lack of accurate measurement of urine volume in rats. It is suggested that, for the sake of uniformity of data on the first day, their differences with other days should be used and used more accurately to measure the exact volume of urine from the newest and updated metabolic cages.

Conclusion

The results of this study concluded that injection of DOX result in reduction of antioxidant concentrations and increased levels of oxidant in the kidney. The use of hydro alcoholic extract of PM, especially its high concentration, significantly improved the above biochemical parameters in addition to renal function which are comparable to vitamin E as an antioxidant.

Acknowledgments

The authors would like to thank Miss Azita Aghaei for their support from Pharmacological Research Center of Medicinal Plants, Mashhad University of Medical Sciences, Mashhad, Iran.

Conflict of interest

The authors declare that they have no conflict of interests.

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