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Isfahan University of Medical Sciences

Original Article

Protective effects of the *Morus alba* L. leaf extracts on cisplatin-induced nephrotoxicity in rat

M. Nematbakhsh[1,2], V. Hajhashemi[3,*], A. Ghannadi[4], A. Talebi[5] and M. Nikahd[3]

[1]*Water and Electrolytes Research Centre, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.*
[2]*Department of Physiology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.*
[3]*Department of Pharmacology and Isfahan Pharmaceutical Sciences Research Center, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.*
[4]*Department of Pharmacognosy, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.*
[5]*Department of Clinical Pathology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.*

Abstract

Cisplatin (CP) as an important anti-tumor drug causes nephrotoxicity mainly by oxidative stress and renin-angiotensin system (RAS). Since flavonoids have high antioxidant activity and probable role in the inhibition of RAS, this study was designed to investigate the protective effect of hydroalcoholic extract and flavonoid fraction of *Morus alba* leaves on cisplatin-induced nephrotoxicity in rat. Extracts of *Morus alba* leaves were prepared and analyzed Phytochemically. Male rats (160-200 g) were used in this study (n=7-9). Normal group received 0.2 ml normal saline intraperitoneally (i.p.) once daily for ten days. Control animals received CP on the third day and saline in the remaining days. Other groups received either hydroalcoholic extract (200, 400 and 600 mg/kg, i.p.) or flavonoid fraction (50, 100 and 200 mg/kg, i.p.) for two days before CP administration and thereafter until tenth day. Serum concentrations of blood urea nitrogen (BUN), creatinine (Cr) and nitric oxide were measured using standard methods. Also left kidneys were prepared for pathological study. The serum levels of BUN and Cr increased in animals received CP. Hydroalcoholic extract was ineffective in reversing these alterations but flavonoid fraction (50 and 100 mg/kg) significantly inhibited CP-induced increases of BUN and Cr. None of the treatments could affect serum concentration of nitric oxide. Flavonoid fraction could also prevent CP-induced pathological damage of the kidney. It seems that concurrent use of flavonoid fraction of *Morus alba* with CP can protect kidneys from CP-induced nephrotoxicity.

Keywords: *Morus alba*; Cisplatin; Nephrotoxicity; Flavonoid fraction

INTRODUCTION

Cisplatin (CP) is an important drug in the treatment of solid tumors, but nephrotoxicity as a side effect limits its use. Nephrotoxicity, as a result of proximal tubule injury, occurs with a high prevalence in the initial days of CP chemotherapy. Recent studies reported that the mechanism of CP nephrotoxicity might be related to oxidative stress, apoptosis or an inflammatory response (1-3) and therefore several chemical and natural compounds with antioxidant and/or anti-inflammatory activity have been examined for their protective effects against CP-induced nephrotoxicity (4). It has also been reported that activation of the renin-angiotensin system (RAS) causes oxidative stress and inhibitors of RAS may improve renal function in CP-treated rats (5-7). Quercetin as a well known flavonoid has been shown to inhibit RAS and oxidative stress (8,9) and as a result limits nephrotoxicity of CP (10,11). Several flavonoids including quercetin and some other polyphenols are found in *Morus alba* leaves (12). *Morus alba* L. or white mulberry (Toote Sefid in Persian) is a small sized tree from Moraceae family. It is widely distributed and cultivated in Iran, India, China, southern Europe and North America (13,14). Its dried or fresh berries as functional berries are eaten and very popular in traditional and folk Iranian medicine (13,15).

*****Corresponding author: V. Hajhashemi, this paper is extracted from the Pharm.D thesis No. 390183
Tel. 0098 311 7922630, Fax. 0098 311 6680011
Email: vhajhashemi@gmail.com

Its leaves in the forms of infusion and decoction are well known in different parts of the world and Iran and used for preventing or treating of some metabolic (13,14), respiratory, urinary and rheumatic disorders (14,15) like diabetes, hypertension and arthritis (16,17). The chemical composition of *M. alba* has been the subject of several experiments. Flavonoids and other polyphenols (12), stilbenes and benzofuran derivatives, alkaloids (14,17), polysaccharides, proteins, minerals (13,17), vitamins and organic acids (17) are the most important components of its leaves. Several pharmacological, biological and clinical properties including antibacterial, antiviral (12), antitussive, hypoglycemic (16,18), hypotensive (13), antiatherogenic, antihyperlipidemia (16,17), diuretic, astringent, antioxidant (14,19) and α-amylase inhibitory effects (16) have been reported for *M. alba* leaves.

MATERIALS AND METHODS

Pharmacognosy

Plant material

The leaves of *M. alba* were collected from Najafabad located in Isfahan province of Iran in May 2011. The plant material was confirmed by Dr. Iraj Mehregan in the Herbarium Department of Science and Research Branch, Tehran Islamic Azad University, Tehran, Iran. The voucher specimen of *M. alba* is in the herbarium department of Isfahan University, Isfahan, Iran with the number of 15619.

Preparation of extracts

The plant extracts were prepared as described before with minor modifications (20,21). Using percolation method and ethanol:water mixtures (70:30 v/v) as the extracting solvent, total hydroalcoholic extract of dried and powdered *M. alba* leaves was obtained after 72 h.

For preparation of the flavonoid-rich extract, *M. alba* leaves were extracted by ethanol:water (90:10 v/v) and ethanol:water (50:50 v/v) in two non-continuous steps for 72 h separately. The extracts were combined and evaporated and the consequent extract was cleared of rubbish non-polar compounds by extraction with chloroform in a separating funnel for several times to produce an aqueous solvent-extracted layer, containing the flavonoid-rich compounds. Hydroalcoholic and flavonoid-rich extracts were filtered and evaporated separately in a rotovapor under reduced pressure and then freeze-dried until dry powders yielded.

Phytochemical screening of different extracts

The different qualitative and preliminary micro-chemical tests are to be performed for establishing profile of the *M. alba* leaves extracts for its nature of chemical constituents. The phytochemical experimental tests were achieved to detect the presence of alkaloids, anthraquinones, tannins, cardiac glycosides, essential oils, flavonoids and saponins in the mulberry leaves (22,23).

The extracts were then analyzed by thin layer chromatography (TLC). The analysis was carried out on aluminum silica gel 60 F_{254} coated plates, with the layer thickness of 250 μm (Merck, Darmstadt, Germany) and developed in different systems where ethyl acetate:formic acid:glacial acetic acid: purified water (100:11:11:26, v/v/v/v) gave the highest resolution. All of the TLC solvents were of analytical grade and Merck brand. The bands were visualized under UV light and natural product reagent (24,25). The identified flavonoid is presented in results section.

Total phenol assay of the extract

Phenolic compounds in the mulberry leaves were assayed by Folin-Ciocalteau method previously described (21,26). Results are given as gallic acid equivalent (GAE) per gram of the lyophilized powder.

Pharmacology

Chemicals

Cisplatin was purchased from Sigma Chemical Company (St. Louis, MO, USA). BUN and Cr kits (Pars Azmoon, Iran) and nitric oxide kit (Promega, USA) were used for biochemical assays.

Animals

Male Wistar rats weighing 160-200 g, were used. The animals were fed with rat chaw and tab water *ad libitum* and kept in a temperature of 22 ± 3°C with a 12 h light/dark cycle. All procedures were approved by the Ethical Committee of the Isfahan University of Medical Sciences, (Isfahan, Iran) and conducted in accordance with the internationally accepted principles for laboratory animal use and care.

After one-week adaptation with the environmental conditions, orbital sinus blood sampling was performed for determination of the baseline values of biochemical parameters. The samples were centrifuged at 1000 rpm for 20 min, then the serum removed and stored at -20ºC until analysis.

Experimental procedure

Rats were randomly divided into groups of 7-9 in each. One group received normal saline (0.2 ml, i.p) from the beginning to tenth day (end of the study). The second group received saline (0.2 ml, i.p.) for 2 days and in the third day CP (7 mg/kg, i.p) was injected. Other groups received either hydroalcoholic extract (200, 400 and 600 mg/kg, i.p.) or flavonoid fraction (50, 100 and 200 mg/kg, i.p.) for two days before CP administration and thereafter until the tenth day. In the last day, rats were anesthetized with ketamine (100 mg/kg) and then sacrificed. Blood samples were collected, centrifuged at 1000 rpm for 20 min, serum was removed and stored at -20ºC until analysis. Both kidneys were removed and weighed. The left kidneys were prepared for pathological examinations.

Measurement of biochemical parameters

Serum creatinine concentration was measured by the Jaffe method and BUN (blood urea nitrogen) by Berthelot method using commercial kits. Serum nitric oxide was measured by a quantitative diagnostic kit (a colorimetric method). Osmolality of serum samples was determined by an Osmometer.

Histopathological examination

The left kidneys of animals halved through a coronal section after removal from the body. Then the two halves were fixed by a 10% solution of formalin for several days. After processing, they were embedded in paraffin and cut into 3-4 micrometer slices. The slices were mounted on glass slides and stained with hematoxylin and eosin (H & E) for light microscopic analysis. The assessment was conducted by a pathologist in a blinded way. The pathologic changes of the kidneys were recorded using a grading scale of 0-4 which was based on a subjective impression of the extent of cortical changes as follows:

0 = indistinguishable from controls
1 = minimal, ≤ 25% cortex affected
2 = mild, > 25% and ≤ 50% cortex affected
3 = moderate, > 50% and ≤ 75% cortex affected
4 = severe, >75% cortex affected

This grading scale is adapted from Goering and coworkers (27) with minor modification.

Statistical analysis

The analysis of data was performed with SPSS statistical analysis software (version 15.0). Data was reported as the mean ± SEM. All results except for pathologic findings were analyzed using one way analysis of variations (ANOVA) followed by Tukey post hoc test. Pathological data were analyzed by Kruskal–Wallis method followed by Mann-Whitney U test. The level of significance was considered as $P<0.05$.

RESULTS

Pharmacognosy

Hydroalcoholic and flavonoid-rich extracts gave dry powders yielded 21.9% and 10.7 % (w/w) respectively. Powders were stored in a refrigerator. The *M. alba* leaves extract was subjected to phytochemical screening for preliminary detection of its natural active constituents. The result of phytochemical screening of extracts revealed that flavonoids and tannins were present in *M. alba* leaves.

TLC separations were used to identify some more flavonoid components in the mulberry leaves. Quercetin as the most important part of the mulberry leaves showed a bright spot in the top of chromatogram (R_f=0.92).

The total phenols determined by Folin-Ciocalteau method showed 440 mg GAE per gram of the lyophilized powder.

Pharmacology

Treatment of animals with CP significantly increased serum concentrations of BUN and Cr. Hydroalcoholic extract (200-600 mg/kg, i.p.) could not reverse the elevation of these biochemical parameters (Figs. 1, 2). Flavonoid fraction at doses of 50 and 100 mg/kg inhibited CP-induced increases of BUN and Cr (Figs. 3,4).

As it is seen in Table 1, both hydroalcoholic extract and flavonoid fraction could not change serum osmolality and nitric oxide concentration.

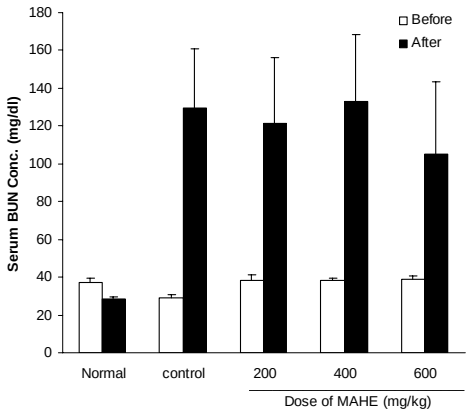

Fig. 1. Effect of different doses of hydroalcoholic extract of *M. alba* on serum BUN concentration in cisplatin-treated rats. Normal group received normal saline (0.2 ml, i.p.) once daily for 10 days. Control group received saline (0.2 ml, i.p.) for 2 days and in the third day cisplatin (7 mg/kg, i.p) was injected. Other groups received hydroalcoholic extract (200, 400 and 600 mg/kg, i.p.) for two days before cisplatin administration and thereafter until tenth day. Data represent mean ± S.E.M of serum Blood urea nitrogen (BUN) concentration of 7-9 animals in each group. MAHE; *M. alba* hydroalcoholic extract.

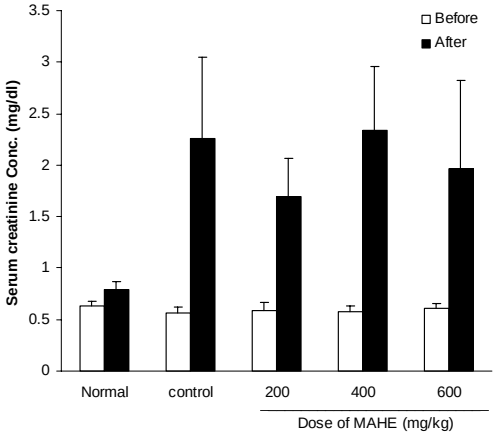

Fig. 2. Effect of different doses of hydroalcoholic extract of *M. alba* on serum creatinine concentration in cisplatin-treated rats. Normal group received saline (0.2 ml, i.p.) once daily for 10 days. Control group received saline (0.2 ml, i.p.) for 2 days and in the third day cisplatin (7 mg/kg, i.p) was injected. Other groups received hydroalcoholic extract (200, 400 and 600 mg/kg, i.p.) for two days before cisplatin administration and thereafter until tenth day. Data represent mean ± S.E.M of serum creatinine concentration of 7-9 animals in each group. MAHE; *M. alba* hydroalcoholic extract.

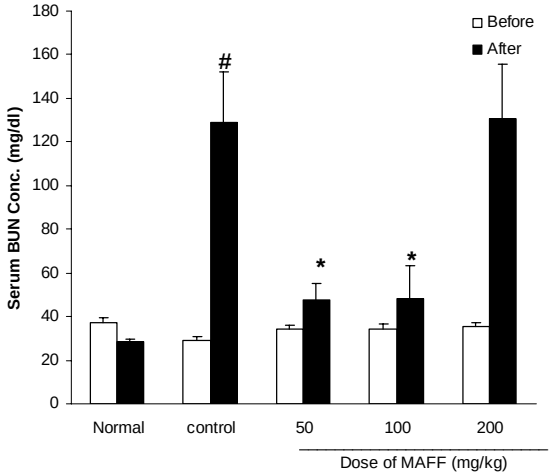

Fig. 3. Effect of different doses of flavonoid fraction of *M. alba* on serum BUN concentration in cisplatin-treated rats. Normal group received saline (0.2 ml, i.p.) once daily for 10 days. Control group received saline (0.2 ml, i.p.) for 2 days and in the third day cisplatin (7 mg/kg, i.p) was injected. Other groups received flavonoid fraction (50, 100 and 200 mg/kg, i.p.) for two days before cisplatin administration and thereafter until tenth day. Data represent mean ± S.E.M of serum BUN concentration of 7-9 animals in each group. MAFF; *M. alba* flavonoid fraction. * $P < 0.05$ compared to cisplatin (control) group; # $P < 0.01$compared to normal group.

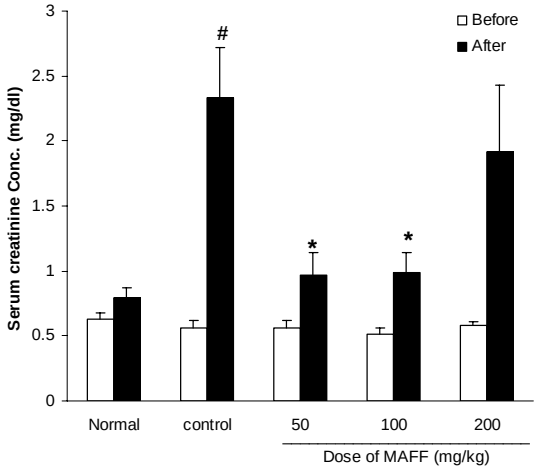

Fig. 4. Effect of different doses of flavonoid fraction of *M. alba* on serum creatinine concentration in cisplatin-treated rats. Normal group received saline (0.2 ml, i.p.) once daily for 10 days. Control group received saline (0.2 ml, i.p.) for 2 days and in the third day cisplatin (7mg/kg, i.p) was injected. Other groups received flavonoid fraction (50, 100 and 200 mg/kg, i.p.) for two days before cisplatin administration and thereafter until tenth day. Data represent mean ± S.E.M of serum creatinine concentration of 7-9 animals in each group. MAFF; *M. alba* flavonoid fraction. $P < 0.05$ compared to cisplatin (control) group; # $P < 0.01$compared to normal group.

The weight of rat kidneys received CP was significantly greater than those of normal rats. Treatment of animals with different doses of hydroalcoholic extract or flavonoid fraction of *M. alba* leaves could not prevent the increase of CP-induced kidney weight. The pathological findings clearly showed that CP significantly damaged the kidney tissue and only flavonoid fraction could improve the CP-induced pathological scores (Table 2 and Fig. 5).

Table 1. Effect of different doses of *M. alba* hydroalcoholic extract and flavonoid fraction on kidney weight , serum osmolality and nitric oxide (NO) concentration in male rats.

Group	Dose (mg/kg)	Kidney weight (g/100 g BW)	Serum osmolality (mM/L)	Serum NO concentration (µM/L)
Normal	-	0.310±0.01	271.0±5.3	8.92±1.95
Control	-	0.495±0.04	324.6±25.3	16.58±2.14
MAHE	200	0.487±0.04	299.4±9.4	21.63±8.43
MAHE	400	0.560±0.05	342.7±25.7	12.39±2.70
MAHE	600	0.424±0.03	311.1±25.4	16.74±2.74
MAFF	50	0.436±0.03	286.6±12.9	16.90±4.34
MAFF	100	0.427±0.04	296.0±8.8	23.95±3.78
MAFF	200	0.477±0.05	301.3±18.2	21.85±4.95

Animals of all groups except normal group received cisplatin (7 mg/kg , i.p.). MAHE; *Morus alba* hydroalcoholic extract , MAFF; *M. alba* flavonoid fraction.

Table 2. Effect of different doses of *M. alba* hydroalcoholic extract and flavonoid fraction on pathological scores

Group	Pathologic grading					
	0	1	2	3	4	n
Normal	7					7
Control (only CP) #		1	4	2	1	8
CP + MAHE (200 mg/kg)		1	5	1		7
CP + MAHE (400 mg/kg)	1		2	4		7
CP + MAHE (600 mg/kg)	2	2	2	1		7
CP + MAFF (50 mg/kg) *	2		6			8
CP + MAFF (100 mg/kg) *	4	1	3			8
CP + MAFF (200 mg/kg)	2	1	3	1		7

The numbers in the table are representative of the numbers of kidneys in each grade of a total of n in each group. Grading scale is as follows: 0 = indistinguishable from controls; 1 = minimal, ≤ 25% cortex affected; 2 = mild, > 25% and ≤ 50% cortex affected; 3 = moderate, > 50% and ≤ 75% cortex affected; 4 = severe, > 75% cortex affected. CP; cisplatin, MAHE; *M. alba* hydroalcoholic extract, MAFF; *M. alba* flavonoid fraction. $P<0.05$ compared to control group and # $P<0.01$ compared to normal group.

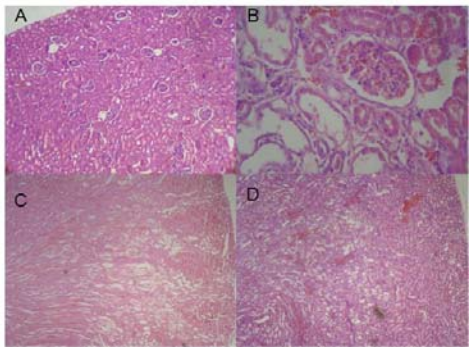

Fig. 5. Effect of *M. alba* polyphenolic extract on the kidney histological changes induced by cisplatin in rat. A: normal group; B: cisplatin-treated group; C: *M. alba* flavonoid fraction (100 mg/kg) + cisplatin; D: *M. alba* flavonoid fraction (200 mg/kg) + cisplatin. Cisplatin treatment was associated with a loss of tubular architecture, and the integrity and hypercellularity of the interstitium (panel B). In contrast, *M. alba* flavonoid fraction prevented the damage (C and D).

DISCUSSION

Cisplatin nephrotoxicity is very complex and several mechanisms including accumulation of the drug in renal epithelial cells, attack of the drug to nuclear and mitochondrial DNA, initiation of a severe inflammatory response, induction of oxidative stress have been proposed (4). Based on these pathways, several *in vitro* and *in vivo* researches have been performed and a relatively large number of chemicals and natural compounds have been tested for their potential protection against CP-induced nephrotoxicity (4).

In the present study, we attempted to investigate the effect of hydroalcoholic and flavonoid rich extracts of *M. alba* leaves on CP- induced nephrotoxicity in rats. Our results clearly showed that flavonoid fraction of *M. alba* inhibited the CP-induced increases of kidney damage biomarkers (BUN and creatinine). Pathological findings also confirmed the protective effect of the flavonoid fraction. Flavonoid fraction showed significant protective effect at doses of 50 and 100 mg/kg and unexpectedly the dose of 200 mg/kg was ineffective. It seems that the dose of *M. alba* flavonoid fraction is detrimental and higher doses lose activity. At present we do not have explanation for it and further studies are required to find out the reason. According to the previous studies and our results in the current study, the leaves of *M. alba* are good source of flavonoid compounds such as quercetin. The findings of many researches confirmed that these compounds are responsible for useful effects in oxidative stress (18,28). On the other hand, several studies reported that the mechanism of CP nephrotoxicity can be associated with oxidative stress (1-4). As a result, the protective effect of flavonoid fraction of *M. alba* on CP-induced nephrotoxicity might be partly due to antioxidant potential of these compounds. Some investigators have reported that CP-induced nephrotoxicity has an inflammatory component and pro-inflammatory cytokines are involved in its pathogenesis. Flavonoid extracts of many plants have shown anti-inflammatory activity (29-31). Consistent with this idea, Choi and Hwang (32) reported that *M. alba* leaf extract reduced production of PGE2, nitric oxide and also TNF-alpha in a mouse macrophage cell line and since these mediators are involved in inflammatory process, it seems that at least a part of the protective effect of *M. alba* leaf extract might be due to its anti-inflammatory potential. Our results also showed that the protective effect of flavonoid fraction is not related to changes in serum concentration of nitric oxide.

CONCLUSION

In conclusion the mechanism of CP-induced nephrotoxicity is very complex and this drug affects several pathways in kidney cells. On the other hand, plant flavonoids also show anti-inflammatory, anti-apoptotic, antioxidant and several other biological activities. Based on these facts, further studies are needed to find out the exact mechanism of the protective effect of *M. alba* leaf extract against CP-induced nephrotoxicity.

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