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Optimization of extraction of *Eucommia ulmoides* polysaccharides by response surface methodology

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ABSTRACT

In this study, extraction yield of *Eucommia ulmoides* polysaccharides was optimized by the utilization of response surface methodology (RSM). Based on contour plots and variance analysis, optimum operational conditions for maximizing extraction yield were found to be extraction time 80 min, ratio of water to raw material 3, and extraction number 3. Then, we investigated the protective effect of the *E. ulmoides* polysaccharides on the tissue peroxidative damage and abnormal antioxidant levels in ischemia reperfusion (IR) induced renal toxicity in male albino rabbits. Decrease in all the enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR)) and non-enzymatic antioxidant (*glutathione* (GSH)), along with an increase in the lipid peroxidative index (malondialdehyde) was found in all the renal ischemia reperfusion (RIR) rabbits as compared with normal controls. The findings indicate that the extract of *E. ulmoides* polysaccharides can protect the kidney against IR induced oxidative damage in rabbits.

1. Introduction

The kidney plays a central role in the regulation of salt and water balance in the body. Disordered regulation of transport in the kidney is responsible for altered salt and water balance in severe pathophysiological states including acute renal failure (ARF). Renal ischemia-reperfusion injury (IRI) is associated with a high level of mortality and morbidity (Bonventre & Weinberg, 2003; Dagher, 2004), and no specific treatment is presently available because of the poor understanding of the pathophysiolology. Although the pathophysiology of AKI is complex and the aetiologies diverse, there is now accumulating evidence to indicate that hypotension, hypoperfusion, hypoxia, oxidative stress and renal vasoconstriction contribute to the pathogenesis (Kunzendorf, Haase, Rolver, & Haase-Fielitz, 2010). IRI is consecutive to a cascade of cellular events including the release of ROS, cell apoptosis, necrosis, infiltration by inflammatory cells, and the release of active mediators leading to tissue damage (Friedewald & Rabb, 2004; Kieran & Rabb, 2004). Restoring the blood flow, although critical to prevent ongoing injury, paradoxically potentiates the deleterious cascade and aggravates the pre-existing damage (Aydin, van Zonneveld, de Fijter, & Rabelink, 2007).

According to the ancient writing of Chinese medicinal herbs (Hu, 1996), *Eucommia ulmoides Oliv.* is commonly used as a tonic for the liver and kidney, thus improving detoxification (by liver) and circulation (via kidney), respectively. The antioxidant activity of *E. ulmoides* leaves has been demonstrated (Hsieh & Yen, 2000; Yen & Hsieh, 1998). Antioxidative flavonoids (rutin, chlorogenic acid, ferulic acid, and caffeic acid) were reported to be contained in *E. ulmoides* leaves (Kulomaa, Siren, & Riekkola, 1997). Antioxidants can inhibit oxidative glycation (glycoxidation) of tissue proteins with reducing sugars (Yamaguchi, Ariga, Yoshimura, & Nakazawa, 2000). The antioxidant effect of some of the chemical constituents of eucommia leaf and bark may also contribute to its antiinflammatory action (Deyama et al., 1988).

The present study was designed to evaluate the antioxidant action of polysaccharides extract of the *E. ulmoides* in rabbits.

2. Material and method

2.1. Extraction of E. ulmoides polysaccharides

The powder of *E. ulmoides* (500 g) was extracted several times with 5 volumes of distilled water at 100 °C for 20–30 min each time. After vacuum filtration, the aqueous extracts were combined and concentrated to one-third of its total volume in vacuum. The resulting concentrated liquor was mixed with three times of its volume of absolute ethanol, stirred vigorously and left overnight at

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4 °C. The precipitated polysaccharides were centrifuged at 8000 × *g* for 30 min and the supernatant discarded. The precipitate of crude polysaccharides was dried at 65 °C to a constant weight and the polymer was weighted by a scale (Sartorious ALC-110.4, Germany). The product yield was measured at the (w/w) % of polysaccharides.

2.2. Optimization of extraction procedure with RSM

A three-level and three-variable of Box–Behnken design (BBD) was applied to optimizing the processing parameters. Three independent variables studied were extraction time (min, *A*), ratio of water to raw material (*B*) and extraction number (*C*), and each variable parameter was set for three variable levels. Totally, seventeen experiments were designed to perform. The extraction yield (%) of polysaccharides was taken as the response (*R*). Regression analysis was performed to establish an empirical second-order polynomial model:

$$R = \beta 0 + \beta 1A + \beta 2B + \beta 3C + \beta 12AB + \beta 13AC + \beta 23BC + \beta 11A^2 + \beta 22B^2 + \beta 33C^2 \quad (1)$$

where *R* = estimate response; β0 = constant; β1, β2 and β3 = linear coefficients; β12, β13 and β23 = interaction coefficients between the three factors; β11, β22 and β33 = quadratic coefficients.

A multiple regression analysis was performed to obtain the coefficients and the equation, which can be used to estimate the response. A Box–Behnken experimental design was used in this study. It is important to note that all interactions higher than second order have been neglected in Eq. (1). A total of 17 experiments were needed to estimate of the model.

2.3. Animals

Healthy adult rabbits procured from experimental animal center, China were used for the present study. The rabbits were provided with standard pelleted rabbit feed and water ad libitum. They were acclimatized to the laboratory conditions and maintained under 12 h light and dark cycles at 25 °C. The experiments were carried out in accordance with the guidelines provided by the Institutional Animal Ethical Committee of China.

Animals were randomly divided into three groups: normal group, renal ischemia reperfusion (RIR) group and EUP-treated group (orally fed with *E. ulmoides* polysaccharides (300 or 600 mg/kg) for 15 days. Then, animals in RIR group and EUP-treated group received renal ischemia and reperfusion operation.

Table 1
Box–Behnken design matrix of real and coded values along with experimental and predicted values for extraction yield (%) of *E. ulmoides* polysaccharides.

Run	A	B	C	Extraction yield (%)
1	−1.00 (60)	−1.00 (3)	0.00 (2)	18.9
2	1.00 (80)	−1.00	0.00	20.7
3	−1.00	1.00 (5)	0.00	18.6
4	1.00	1.00	0.00	23.8
5	−1.00	0.00 (4)	−1.00 (1)	17.3
6	1.00	0.00	−1.00	21.5
7	−1.00	0.00	1.00 (3)	22.1
8	1.00	0.00	1.00	24.5
9	0.00 (70)	−1.00	−1.00	19.4
10	0.00	1.00	−1.00	20.3
11	0.00	−1.00	1.00	21.8
12	0.00	1.00	1.00	20.5
13	0.00	0.00	0.00	23.3
14	0.00	0.00	0.00	23.8
15	0.00	0.00	0.00	23.1
16	0.00	0.00	0.00	22.9
17	0.00	0.00	0.00	23.5

2.4. Induction of acute renal injury

Surgical operation was performed under intraperitoneal sodium pentobarbital anesthesia. For the renal ischemia, the right kidney was removed 3 weeks before the ischemia. Left renal ischemia was conducted for 45 min of occlusion of the renal artery and vein followed by 24 h of reperfusion. Occlusion was verified visually by change in the color of the kidneys to a paler shade and reperfusion by a blush.

2.5. Biochemical analysis

Cr, IL-6 and bFGF levels were measured with commercially available kits.

The tissues MDA concentration was determined using the method described by Jain, McVie, Duett, & Herbst (1989) based on TBA reactivity. The tissues GSH concentration was measured using the method described by Beutler, Dubon, & Kelly (1963). GSH-Px activity was assayed according to Paglia and Valentine (1967) based on that of GSH-Px catalyses the oxidation of GSH by Cumene Hydroperoxide. SOD activity was measured at 505 nm by calculating inhibition percentage of formazan dye formation. The activity of catalase was measured according to the method of Aebi (1974). GR

Table 2
ANOVA for Response Surface Quadratic Model analysis of variance.

Source	Sum of squares	df	Mean square	*F* value	*p*-value Prob > *F*	
Model	66.91	9	7.43	10.07	0.0030	significant
A–A	23.12	1	23.12	31.32	0.0008	
B–B	0.72	1	0.72	0.98	0.3563	
C–C	13.52	1	13.52	18.31	0.0037	
AB	2.89	1	2.89	3.91	0.0884	
AC	0.81	1	0.81	1.10	0.3297	
BC	1.21	1	1.21	1.64	0.2413	
A^2		4.09	1	4.09	5.53	0.0509
B^2		14.18	1	14.18	19.20	0.0032
C^2		4.09	1	4.09	5.53	0.0509
Residual	5.17	7	0.74			
Lack of Fit	4.68	3	1.56	12.79	0.0162	significant
Pure Error	0.49	4	0.12			
Cor Total	72.08	16	R-Squared			
Std. Dev.	0.86		Adj R-Squared		0.9283	
Mean	21.53		Pred R-Squared		0.8361	
C.V. %	3.99		Adeq Precision		−0.0495	
PRESS	75.64		R-Squared		9.105	

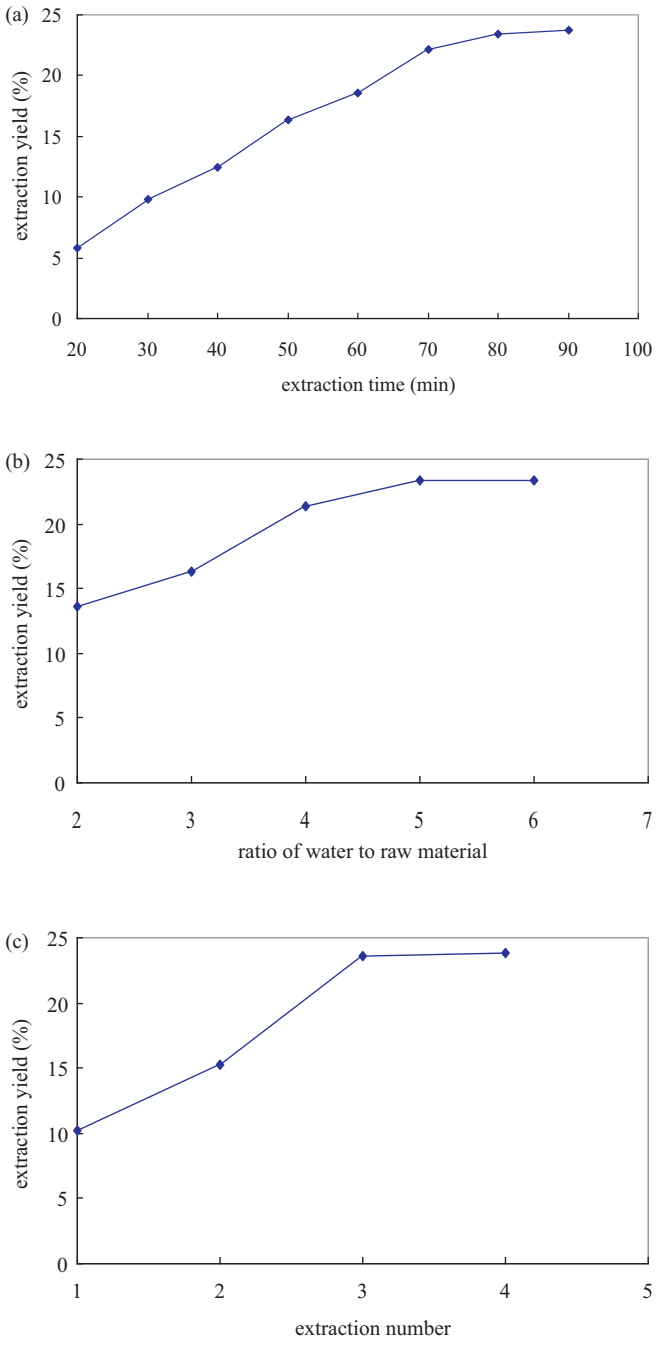

Fig. 1. Effect of extraction time (a), ratio of water to raw material (b) and extraction number (c) on extraction yield.

activity was assayed according to Carlberg and Mannervik (1975) as the decrease in absorbance of NADPH at 340 nm.

2.6. Histopathological studies

The kidneys were removed from the animals and the tissues were fixed in 10% formalin for at least 24 h. Then the paraffin sections were prepared (Automatic tissue processor, Autotechnique) and cut into 5 lm thick sections in a rotary microtome. The sections were then stained with hematoxylin-eosin dye and were studied for histopathological changes.

Table 3
Effect of *E. ulmoides* polysaccharides on serum Cr, renal IL-6 and bFGF levels.

Group	Cr (μmol/L)	IL-6 (pg/g)	bFGF (ng/g)
NC	70.72 ± 4.01	212.29 ± 12.42	21.17 ± 1.82
RIR	101.01 ± 8.74[a]	481.63 ± 31.26[a]	13.42 ± 1.96[a]
EUP (300 mg/kg)	86.04 ± 6.08[b]	412.35 ± 38.94[b]	16.94 ± 1.03[b]
EUP (600 mg/kg)	74.09 ± 5.09[b]	305.28 ± 27.02[b]	20.35 ± 2.52[b]

[a] $p < 0.01$, compared with NC group.
[b] $p < 0.01$, compared with RIR group.

2.7. Statistical analysis

All values are expressed as mean ± S.D. The significance of differences between the means of the treated and untreated groups has been calculated by unpaired Student's *t*-test, and *p*-values lesser than 0.05 were considered significant.

3. Results and discussion

3.1. Single-factor test

As shown in Fig. 1(a), when extraction time increased from 20 min to 80 min, extraction yield increased from 5.7% to 23.6%. After that, extraction yield no longer significantly increased. The effect of ratio of water to raw material on the extraction yield was shown in Fig. 1(b). With the increase of ratio of water to raw material from 2 to 5, the extraction yield increases rapidly from 13.4% to 23.5%. The effect of extraction number on the extraction yield was shown in Fig. 1(c). With the increase of extraction number from 1 to 3, the extraction yield increases rapidly from 9.8% to 24.1%.

3.2. Fitting the process models

The set of all the experiments performed and their corresponding predicted values for the extraction yield (%) are given in Table 1. Experimental data for extraction yield was fitted to the second order polynomial equation ($R^2 = 0.9283$), indicating that 92% of variability in a response could be explained by the developed RSM model. The experimental results were analyzed by using ANOVA (analysis of variance) for extraction yield and cited in Table 2. *F* value is an indication of the level of significance. The high *F* value indicates that a factor has higher significant effect on the corresponding response. A model *F* value is calculated as $F = \text{MSF/MSE}$, where MSF and MSE are mean squares of factors and mean squares of errors, respectively (Alyani, Towfighi, & Sadrameli, 2011). The *p* values were used as a tool to check the significance of each of the coefficients which, in turn, are necessary to understand a pattern of mutual interactions between the test variables. Smaller the magnitude of p value, more significant is the corresponding co-efficient (Hanchinal, Survase, Sawant, & Annapure, 2008). The values of *p* less than 0.05 indicate that model terms are significant. Table 2 show that a model or intercept *p* value is very low ($p < 0.005$), which indicates the developed model is significant.

Further, significance of each coefficient was determined by *F* and *p* values. The coefficient estimates and corresponding *p* values

Table 4
Effect of *E. ulmoides* polysaccharides on renal MDA and GSH levels.

Group	MDA	GSH
NC	3.57 ± 0.38	185.37 ± 12.63
RIR	8.35 ± 0.67[a]	103.52 ± 8.52[a]
EUP (300 mg/kg)	6.71 ± 0.54[b]	143.29 ± 11.09[b]
EUP (600 mg/kg)	4.91 ± 0.35[b]	168.03 ± 12.49[b]

[a] $p < 0.01$, compared with NC group.
[b] $p < 0.01$, compared with RIR group.

Table 5
Effect of *E. ulmoides* polysaccharides on renal CAT, SOD, GSH-Px, and GR activities.

Group	SOD	CAT	GSH-Px	GR
NC	275.81 ± 24.15	52.15 ± 4.44	37.04 ± 2.58	41.59 ± 3.04
RIR	159.25 ± 16.08[a]	25.04 ± 2.31[a]	15.37 ± 1.62[a]	23.16 ± 1.59[a]
EUP (300 mg/kg)	190.46 ± 21.11[b]	38.91 ± 2.84[b]	22.18 ± 1.99[b]	32.05 ± 1.95[b]
EUP (600 mg/kg)	251.15 ± 24.38[b]	49.27 ± 3.36[b]	32.05 ± 2.07[b]	38.49 ± 2.88[b]

[a] $p < 0.01$, compared with NC group.
[b] $p < 0.01$, compared with RIR group.

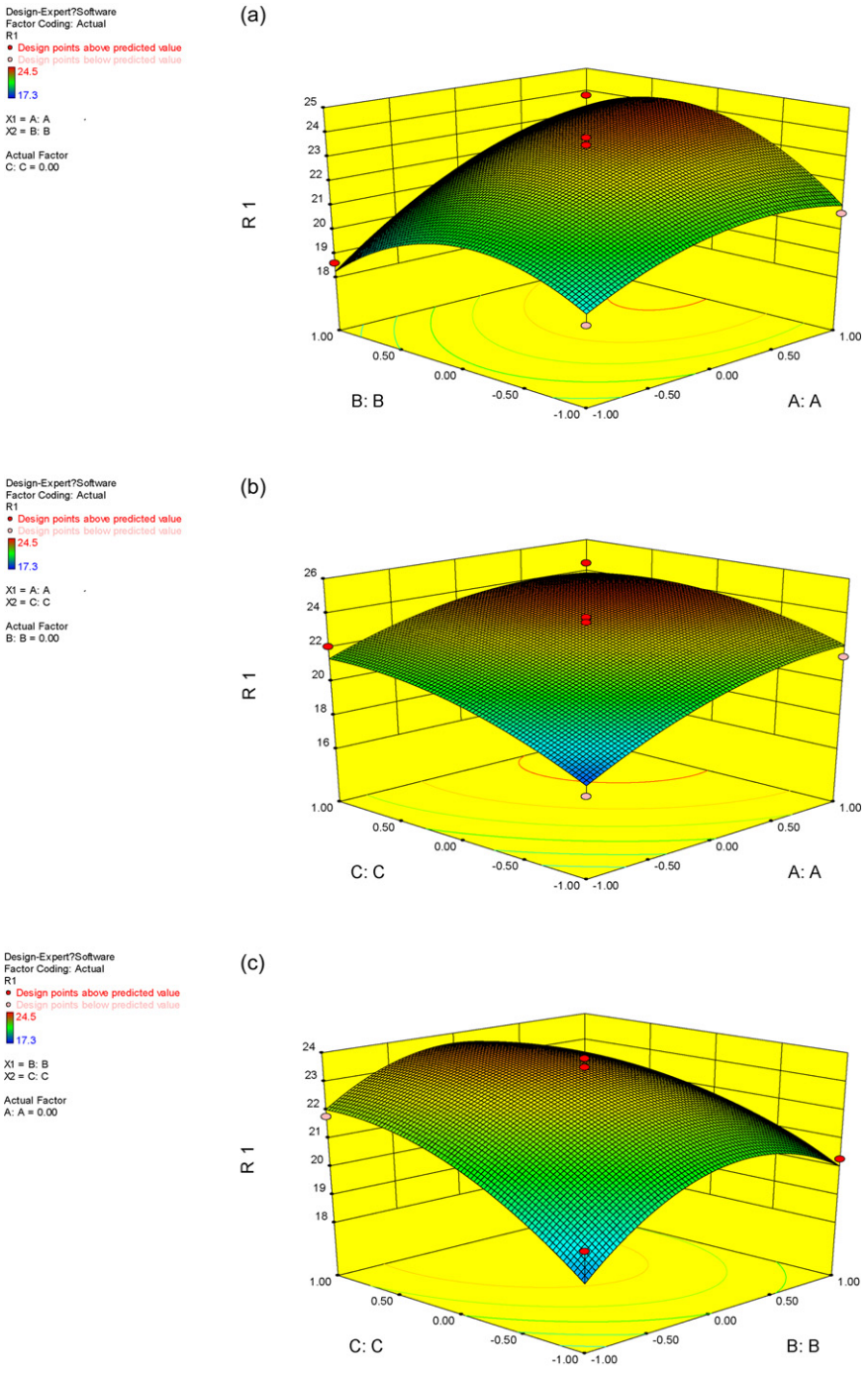

Fig. 2. Response surface diagrams showing the effects of the mutual interactions between two independent variables (a) extraction time and ratio of water to raw material, (b) extraction time and extraction number, (c) ratio of water to raw material and extraction number.

suggest that among the test variables used in this study, A (extraction time), C (extraction number), B^2 (ratio of water to raw material)^2 are significant terms with p values less than 0.05 (Table 3). Extraction time and extraction number ($p < 0.05$) have the highest impact on extraction yield, followed by ratio of water to raw material. The corresponding second order mathematical model for extraction yield that was found after analysis for regression is given as follows:

$$R1 = +23.32 + 1.70 * A + 0.30 * B + 1.30 * C + 0.85 * A * B - 0.45 * A * C - 0.55 * B * C - 0.99 * A2 - 1.84 * B2 - 0.98 * C2$$

Fig. 2a–c shows contour plots and surface plots for extraction percent in corresponding interaction effects. The graphical representation was used to accomplish a better understanding of the interactions between variables and to determine the optimum level of each variable for a maximum extraction yield in the batch system. The results confirmed that extraction time and extraction number were significant for extraction yield. From Fig. 2a–c, it can be seen that the extraction time had positive synergistic effects when coupled with ratio of water to raw material. The singular effect of ratio of water to raw material on extraction yield was negative. In combination as a pair, however, all three intercept terms were not statistically significant in terms of their p value.

Based on contour plots and variance analysis, optimum operational conditions for maximizing extraction yield were found to be extraction time 80 min, ratio of water to raw material 3, and extraction number 3. Under the optimum operating conditions determined, 23.9% extraction yield was achieved.

3.3. Renal protective activity of E. ulmoides polysaccharides

In the present investigation, renal protective activity of *E. ulmoides* polysaccharides was evaluated in RIR rabbits. Oxidative stress has been postulated as a major molecular mechanism involved in experimental animal models. The increased serum biochemical enzymes, such as Cr, IL-6, and bFGF, have been attributed to the damaged structural integrity of the kidney (Oosterwijk et al., 2011). In the present study, the effect of *E. ulmoides* polysaccharides on serum Cr, renal IL-6 and bFGF levels in rabbits is shown in Table 3. RIR rabbits sustained significant renal damage as represented by changes in these biochemical enzymes. Compared with normal control group, there was a significant increase in the serum Cr and renal IL-6 levels and a decrease in renal bFGF level in RIR group. The administration of *E. ulmoides* polysaccharides dose-dependently significantly decreased serum Cr, renal IL-6 levels, and increased renal bFGF levels in EUP groups rabbits compared with the RIR group. This showed that *E. ulmoides* polysaccharides could decrease IR-indiced renal damage in rabbits.

Many studies have shown that the renal protective effects may be related to triterpenoids' exhibiting a membrane-stabilizing capacity (Khoury, Namnesnikov, Fedorov, Abu-Ghazala, & Weinbroum, 2010) or an antioxidant capacity to scavenge reactive oxygen species (Chang et al., 2004). Reactive oxygen species, including superoxide, hydroxyl radicals and hydrogen peroxide, are generated and react with biological molecules, eventually damaging membranes and other tissues (Vuillaume, 1987). Antioxidant enzymes (SOD, GSH-Px and catalase) represent one protection against oxidative tissue-damage. SOD converted O_2 into H_2O_2. GSH-Px and catalase metabolize H_2O_2 to non-toxic products. The results obtained indicate increased MDA levels in the kidney in response to IR treatment, implying increased oxidative damage to the kidney. RIR also caused a decrease in GSH-Px, SOD and catalase activities in the kidney over those of the control group. Under oxidative stress, some endogenous protective factors such as GSH-Px and catalase are activated in the defense against oxidative injury (Kyle, Miccadei, Nakae, & Farber, 1987).

The administration of *E. ulmoides* polysaccharides dose-dependently significant decreased renal MDA level and increased GSH level in EUP groups compared to RIR group (Table 4).

In the present study, oxidative stress induced by IR statistically decreased the activities of antioxidant enzymes including CAT, SOD, GSH-Px, and GR in rabbit kidney (Table 5). Feeding of *E. ulmoides* polysaccharides dose-dependently significantly reversed the IR-induced decreased activities of SOD, CAT, GSH-Px, and GR to normal in kidney in EUP groups. It is possible that the *E. ulmoides* polysaccharides exerts its renal protective activity by, at least partly, its antioxidant and free radical scavenging activities.

In control animals, we demonstrated normal tubular lumen, glomerular and free space in corpuscle. In RIR group, demonstration showed severe tubular dilatation, focal glomerular necrosis, and extent free space in corpuscle, loss of nuclei, predominates over morphological features of apoptosis (chromatin condensation and cell shrinkage, swelling tubular, lumina congestion, severe diffuse interstitial edema and dilatation of tubular structure. The results of histological examinations showed that the induction of EUP leads to the improvement in the condition of renal tubules and glomerulus when compared with IR.

4. Conclusion

The inhibitory effects of *E. ulmoides* polysaccharides may be useful as a renalprotective agent against IR-induced renal oxidative injury in vivo.

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