



The Effect of Pine Cones Aqueous Extract on Renal Function in Male Rats Maasoomeh Rezaei, M.Sc.¹, Neamatollah Razmi, Ph.D.², Ameneh Khoshvaghti, Ph.D.³

1- Department of Biochemistry, Fars Sciences and Researches Branch, Islamic Azad University, Shiraz, Iran

2- Associate Professor, Department of Biochemistry, Shiraz Branch, Islamic Azad University, Shiraz, Iran

3- Associate Professor, Department of Clinical Sciences, School of Veterinary Medicine, Kazerun Branch, Islamic Azad University, Kazerun, Iran

(Corresponding author; E-mail: khoshvaghti2004@yahoo.com)

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Abstract

Background: Antioxidant effects of cypress cones extract have been previously demonstrated. In this study, the protective effect of cypress cones extract was investigated. Gentamicin, an aminoglycoside antibiotic administered for the treatment of gram-negative bacteria infections, was used for nephrotoxicity induction.

Methods: In this study, 60 wistar male rats were randomly divided into the six groups (n=10); control group (C) received nothing, sham group (S) received distilled water, group D received gentamicin for induction of nephrotoxicity, group GE received gentamicin as well as Cypress cones aqueous extract intra- peritoneally for 16 days, group DE received the extract after being sick and group E received just the extract. Sampling was done after 16 and 32 days of study. Sodium and potassium concentrations were measured using flame photometry method. Other parameters were assayed by colorimetric method.

Results: Statistically significant difference was detected between the control group and group D in regard to the mean level of Bun, creatinine, sodium, potassium, calcium and chloride ($P<0.05$), while no statistically significant difference was observed in the mean level of these parameters in DE and GE groups (the groups receiving Gentamicin and pine cones aqueous extract) in comparison with control group in days 16 and 32 ($P>0.05$). In this study, cypress cones aqueous extract could change the increased levels of Bun and serum creatinine and decreased levels of sodium, potassium, calcium and chloride resulted from kidney injury into their natural levels.

Conclusion: The present study revealed that cypress cones extract can improve Gentamicin-induced renal failure in rats. As previous studies have proved the existence of antioxidants in cypress cones, the observed health-promoting effects of aqueous extract can be attributed to these properties.

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Introduction

Cedar tree which is widely found in Mediterranean regions encompasses leaves and cones that had significant role in traditional medicine as astringent, anti-rheumatoid, anti-hemorrhoid and vasoconstrictor agents. Forty nine main compositions have been identified in its oil, including pinene (48.2%) and α -3-carene (19.1%) categorized in the monoterpenes group. Moreover its extract is rich in Thymoquinone (1).

Cupressus sempervirens from Cupressaceae or cypress plant family is a single-based plant with mutually exclusive leaves, mostly small and scaly and needle-shaped. Cones of this plant are oval, leathery, and more or less thick. Scales are not bumper and overlapping and seeds are wingless. In traditional medicine, the cones and leaves of *Cupressus sempervirens* are used as antiseptic, astringent and diuretic agents (1).

Pre-treatment with aqueous extract of this plant cone stimulates zonal angiogenesis and improves the central skin damage caused by anemia (2). The main extracted oil from the cone of the plant depicts its dramatic antioxidant and anti-microbial activities (3). The methanol extract of *Cupressus sempervirens* and its related flavonoids have protective effects against lead acetate poisoning (4) and methanol extract of its cone has anti-microbial and anti-parasitic effects (5).

Cupressus sempervirens and *Juniperus* methanolic extracts have protective properties in liver and kidney damages and may be useful in the treatment of renal and hepatic disorders (6). The extract of this plant cone has a significant impact in reducing lipid in male Wistar rats (7). Its cone oil is useful in

wound healing through increasing collagen and growth of fibrous tissue (8).

Gentamicin is an aminoglycoside antibiotic used in the treatment of Gram-negative bacterial infections, but due to its side effects such as kidney toxicity, its use is limited (9). Through damaging mitochondrial function of tubular cells, interfering with tubular function, high oxidative stress and producing free radicals (10), gentamicin causes necrosis in upper tubes of renal cortex, increase of urea and nitrogen in blood and creatinine concentration in serum and renal toxicity.

Original compounds of the Siberian plant are monoterpenes and Thymoquinone that their antioxidant properties have been proved by previous researchers (1, 3, 11-15). Since there is still no study about the effect of aqueous extract of pine cones on renal toxicity, the present study was designed to determine the impact of cypress cones aqueous extract on Gentamicin-induced renal toxicity in rats.

Materials and Methods

Sixty adult male wistar rats with average weight of 200g were selected and divided into the following groups (n=10):

- 1- Control group (C) received nothing
- 2- Sham group (S) received only distilled water
- 3- Group (D) received Gentamicin for inducing nephrotoxicity
- 4- Group (GE) received Gentamicin and the extract simultaneously
- 5- Group (DE), as Gentamicin-induced Nephrotoxic group, received the extract after being sick

6- Group (E), as healthy experimental group, received only the extract

All ethical points approved by supervising committee of laboratory animals in Kazeroun Azad University were observed.

Renal failure was induced by daily intraperitoneal administration of 100 mg/kg gentamicin (Iran-Alborz) for 16 days (5cc for each rat weighing 200mg) (16). Two days after the last injection, for ensuring of the occurrence of renal failure, blood samples of two randomly selected rats were taken for the measurement of blood urea, nitrogen and creatinine levels.

The groups E, DE and GE received 150 mg / kg pine cones aqueous extract (2cc for each rat weighing 200mg). Sampling was done in days 16 and 32 of the study. Two days after the last injection, blood samples of all groups were taken. First, the rats were anesthetized and then their skin was cut and blood was taken from the heart through a 5 cc syringe. Blood samples were collected into vacutainers and serum was separated by centrifuging at 750 g for 15 min and stored at -20°C until use.

Method of Extraction:

First, cupressusempervirens cone was prepared and then 500 grams of the cone was sliced and chopped, dried in the absence of sun shine or heat and was powdered. Then, 100 grams of the powder was removed and extracted by maceration method. The clear extract obtained by rotary operator after 24 hours was concentrated at 40 to 50° C in a vacuum. To ensure the absence of moisture, the desiccating

device was used for 24 hours and then the dry extract was obtained.

The biochemical parameters were measured using Auto Analyzer set (Germany Ependorf Model) and Pars Azmoon kits. Serum urea was assayed by enzymatic method. Urea nitrogen by conversion of urea to BUN using formula (17), creatinine by modified Jaffe (18), albumin by Bromocresol green, total protein by Biuret (19) and calcium by ortho-cresol phthalein methods were measured. Sodium and potassium were measured by flame photometry method (20, 21) using Krnynyk 410 flame photometer (England).

Chloride was measured through thiocyanate method using a biochemical assay kit and a spectrophotometer. The results were analyzed through SPSS software and using ANOVA, Tukey and Duncan statistical tests (22).

Results

The results of this research have been presented in Tables 1 to 4. As it is seen, there is a statistically significant difference in the mean levels of BUN and creatinine in the 16th and 32nd days between the group D (suffering from renal failure and not receiving extracts) and the control group (P=0.001).

Also, there is a significant difference between group DE (the group that received the extract after induction of renal failure) and group D in days 16 and 32 in mean concentration of BUN and creatinine; in other words, the extract could decrease the BUN and creatinine levels which had been increased in plasma.

As it is seen in table 3, in day 16, a significant difference in calcium level was observed in the control group compared with GE and D groups. This has been due to the decreased

value of this parameter in the experimental groups (GE and D). In days 16 and 32, a statistically significant difference in the mean level of albumin was observed between the control group and GE group which is due to the decrease of albumin in GE group that received the extract and Gentamicin simultaneously (Tables 3 and 4). Moreover, in none of the 16th and 32nd days of study, there was statistically significant difference in the mean level of total protein in the control group compared to the other groups.

Mean level of sodium in day 16 was significantly different among control (C), GE and D groups (Table 3). This difference was resulted from the decrease of sodium concentration in groups GE and D (groups suffering from renal failure). In addition, in day 32, a significant difference

was detected between groups DE and D (group with renal failure), since the extract has caused a reduction in sodium excretion. A statistically significant difference in mean potassium level was observed between the control group and the group with renal failure that had not received the extract (D). The results have been presented in tables 3 and 4.

In day 16, chloride level showed significant difference in the control group compared to GE and D groups (Table 3). There was also a significant difference in chloride level between the control and D groups in day 32 (Table 4). The statistically significant difference between the control group and group D in days 16 and 32 indicates an increase in renal excretion of chloride after receiving gentamicin and the resulted kidney damage.

Table 1. The levels of different biochemical parameters in the 16th day of the study in the studied groups

Group	Parameter	BUN (mg/dl)	Creatinine (mg/dl)	Calcium (mg/dl)	Albumin (g/dl)	Total Protein (g/dl)	Sodium (mEq/L)	Potassium (mEq/L)	Chloride (mEq/L)
E		24.6±4.5	0.7±0.12	20.6±1.03	4.08±0.11	7.4±0.25	153±4.3	6.3±0.31	100.2±1.5
GE		26±5.8	0.88±0.22	17.3±1.3	3.9±0.29	6.7±0.53	149.8±1.9	5.8±0.34	96.4±3.05
D		35.5±5.3	1.14±0.43	19.5±0.85	4.38±0.22	7.46±0.26	146.8±0.84	4.76±0.11	94.6±4.39
DE		26.2±2.39	0.70±0.07	20.9±0.74	4.02±0.08	6.78±0.36	162.6±2.5	6.14±0.21	99±2.12
S		22.8±1.9	0.58±0.13	22.3±0.85	4.24±0.09	7.06±0.24	154.8±1.3	5.82±0.08	102.2±1.3
C		23.2±1.79	0.52±0.04	22.3±0.58	4.30±0.10	7.16±0.30	153.86±1.3	5.64±0.38	102±1.2

Table 2. The levels of different biochemical parameters in the 32nd day of the study in the studied groups

Group	Parameter	BUN (mg/dl)	Creatinine (mg/dl)	Calcium (mg/dl)	Albumin (g/dl)	Total Protein (g/dl)	Sodium (mEq/L)	Potassium (mEq/L)	Chloride (mEq/L)
E		24.8±4.8	0.62±0.11	21.14±0.94	4.06±0.15	6.76±0.21	158.6±0.89	5.68±0.22	99.6±2.07
GE		21.2±1.5	0.68±0.08	21.04±1.05	3.94±0.09	6.68±0.19	156.4±0.55	5.56±0.21	98±1.41
D		35.5±5.26	1.14±0.43	19.52±0.85	4.38±0.22	7.46±0.26	146.8±0.84	4.76±0.11	94.6±4.4
DE		25±4.53	0.72±0.15	20.22±0.53	4.02±0.11	6.7±0.35	155.6±0.55	5.58±0.26	100±0.71
S		22.8±1.90	0.58±0.13	22.3±0.85	4.24±0.09	7.06±0.24	154.8±1.3	5.82±0.08	102.2±1.3
C		23.2±1.8	0.52±0.04	22.3±0.58	4.3±0.10	7.16±0.30	153.86±1.3	5.64±0.38	102±1.2

Table 3. Comparison of experimental groups and the control group in the 16th day of the study

Parameter Group	BUN (mg/dl)	Creatinine (mg/dl)	Calcium (mg/dl)	Albumin (g/dl)	Total Protein (g/dl)	Sodium (mEq/L)	Potassium (mEq/L)	Chloride (mEq/L)
E	0.99	0.76	0.09	0.32	0.78	0.83	0.10	0.87
GE	0.86	0.12	×0.000	×0.006	0.30	0.03	0.99	×0.02
D	×0.001	×0.001	×0.001	0.97	0.72	×0.000	×0.000	×0.001
DE	0.82	0.76	0.19	0.12	0.50	×0.000	0.49	0.44
S	1.00	0.99	1.00	0.99	0.99	1.00	1.00	1.00

*:p<0.05

Table 4. Comparison of experimental groups with the control group in the 32nd day of the study

Parameter Group	BUN (mg/dl)	Creatinine (mg/dl)	Calcium (mg/dl)	Albumin (g/dl)	Total Protein (g/dl)	Sodium (mEq/L)	Potassium (mEq/L)	Chloride (mEq/L)
E	0.98	0.97	0.30	0.09	0.20	×0.000	0.88	0.53
GE	0.95	0.80	0.22	0.003	0.08	0.12	0.42	0.08
D	×0.000	0.001	×0.000	0.93	0.49	×0.000	×0.000	×0.000
DE	0.96	0.62	0.007	×0.003	0.10	0.77	0.50	0.71
S	1.00	0.99	1.00	0.98	1.00	1.00	1.00	1.00

*:p <0.05

Discussion and conclusion

The present study was an attempt to investigate the antioxidant effects of pine cone extract, reported by some previous researchers (1, 3, 9, 10, 13, 15), on gentamicin-induced kidney damage. It was found that pine cone extract causes a reduction in increased plasma concentration of creatinine and urea resulted from kidney disorder. Moreover, it prevents ions excretion caused by gentamicin effect in kidney.

Several mechanisms are involved in the nephrotoxicity induced by gentamicin such as accumulation of lysosomes and oxidative stress. Gentamicin is accumulated in lysosomes after entering the cell through endocytosis and leads to an increase of lysosomal membrane permeability through the

production of reactive oxygen groups (ROS) and in turn, it induces apoptosis in proximal cells of kidney (23,24). Gentamicin, besides having impacts on renal proximal tubular epithelium cells, attributed to an endocytotic receptor as megalin/cubilin complex, through its effect on Glomerule cells causes cells contraction and as a result, it reduces Glomerular filtration coefficient (KF) and decreases Glomerular filtration rate (GFR). The increase in the production of super oxide anion plays a key role in inducing the contraction of mesenchymal cells. Through reduction of GFR, creatinine clearance is reduced leading to accumulation of creatinine and urea in blood.

Increased urea-nitrogen (BUN) and creatinine in blood resulted from gentamicin intake show that gentamicin is nephrotoxic (25- 27). The increase in urea-nitrogen and

creatinine, as the result of gentamicine use, and consequent kidney damage have been reported in similar studies by Ahmadi et al (16).

Since 90% of filtered sodium in the first part of nephron is re-absorbed and potassium re-absorption is fully carried out in the proximal tubes (28, 29), so the reduction of serum concentration of sodium and potassium as well as increased urea and creatinine resulted from renal damage due to gentamicin use is similar to the results reported in the study by Ciric et al. (30).

The marked increase in urinary calcium, in gentamicin-induced kidney damage, can be attributed to the decrease of calcium re-absorption in the distal tubes. So, gentamicin impact on urinary calcium occurs in different parts of the nephron and is not restricted to the proximal nephron (31, 32). In this study, the increase of chloride secretion in the urine and the decrease of its absorption in the blood after gentamicin use were similar to the results obtained in the study by Garry et al. (33).

Statistically significant differences found between the mean values of albumin in different groups compared with control group shows that if the disease and its causes are treated and eradicated, the extract can improve kidney problems leading to the excretion of albumin. But if the disease still exists, protective effects of the extract are less than the pathogenic effects of gentamicin on kidney.

It is worth mentioning that despite the statistically significant differences between GE and C groups, the mean value of albumin in the GE was within the normal range, indicating that even in the group which received gentamicin

and the extract simultaneously, the use of the extract could prevent the drastic reduction of albumin.

The normal range of albumin level in group D (with renal failure) and absence of any statistically significant difference between this group and the control group could be attributed to the severe dehydration of rats in this group keeping albumin in the normal range in a false way.

There were no statistically significant differences in the mean value of total protein in the two studied days among the control group and the other groups, emphasizing that even in the rats with renal failure which experienced no treatment, the decrease of protein was not adequate enough to show statistically significant difference. It should be noted that inflammation can increase globulins which in turn can compensate the impact of reduced albumin on total protein levels.

The main constituents of Siberian cones are δ -3-carene, α -pinene and α -cedrol which make 40.2 to 60 percent of the extract. These compounds are monoterpenes.

The main aglycone in the extract of Siberian pine cone is Thymoquinone and the main compositions are δ -3-carene and α -pinene (1, 9, 10).

Milos et al (1998) examined the composition of Siberian pine cone extract and reported that its main glycans are three hydroxy benzoic acid methyl (15.5%) and Thymoquinone (7.3-7.9 %). The researchers presented the other glycans of pine cone extract as Pryla alcohol (2.8-6.3 %), - the first pi-simin 8 (3.5-4.6%), 2-phenyl ethanol (7.2-9.6%) and carvacrol (3.6-5.2 %) (34).

Thymoquinone has strong antioxidant properties that protect the organs against oxidative damages induced by

generated free radicals (15) and the main components of pine cone extract are also monoterpenes and Thymoquinone. Many studies have been conducted about the role of free radicals in renal injury induced by gentamicin and as it has been reported the use of antioxidants is much effective to improve this type of kidney damage confirming the fact that reactive oxygen species (ROS) play an important role in the development of gentamicin-induced kidney damage (23-25).

On the other hand, gentamicin reduces the activity of enzymes in antioxidant defense system such as superoxide dismutase, peroxides glutathione and Catalase (25, 26).

In all species of *Cupressus Sempervirens*, methanol extract of leaf and fruit showed antioxidant activity when tested by FTC (ferric thiocyanat) and TBA (thiobarbituric acid) (9,27).

Tumen et al. (2012) examined the antioxidant activity of Cones in two different species of cypress and reported that the antioxidant activity of the extracts depends on the applied methods. They stated that their study was the first study on anti-cholinesterase and anti-tyrosinase effects of *Cupressus Sempervirens* measures (13).

Thus, concerning the gentamicin-induced kidney damage resulting from its oxidative effect and based on the investigations carried out previously, the presence of antioxidant compounds in cone pine can be proved. The reduction of the devastating effects of gentamicin on kidney, by using the extract, can be attributed to the antioxidant effects of the extract. This finding is in line with the findings reported by Sana et al (2012). They stated that the Methanol extract of pine (*sempervinescupressus*) and (*Juniperus*) contain flavonoids and phenolic acids which are among the important

antioxidants (6) and that the decreased creatinine and increased urea in blood are due to poisoning correspond with the ccl4. The results are also in line with Corey M. et al (2012), revealing that the extract of *semipervinscupressus* seed (pine seed) prevents liver damage induced by lead acetate because of flavonoids in the extracts (4). Therefore, the cedar cone extracts which contain antioxidants such as TQ, terpenes, tannins and so on (1, 3, 11, 12, 13, 15) can reduce urinary excretion of ions by trapping free radicals and prevent tubular damage. It can also increase creatinine clearance and decrease urea nitrogen in plasma. Ethanol extract of *OceridentalisCassi* also has the same effect as Pine extract (35). Pine cone seeds contain flavonoids, tannins and

Phenols (14). Cedar cone fruits are rich in tannin and monoterpenes hydrocarbon (11).

Emami et al. (2006) stated that oil extract of leaves and fruits of *cupressussempervinesis* has high antibacterial activity and is rich in Sayvny and tannins. It also contains some flavonoids and alkaloids (11).

Methanol extract of Cedar leaf (*cupressussempervines*) and *Juniperus* reduces creatinine and blood urea increased due to ccl4 poisoning (3) because of the presence of flavonoids and phenolic acids, which are important antioxidants.

The content of seed extract (*cupressussempervins*) prevents liver damage induced by acetate lead, due to the presence of flavonoids in the extract (36).

In this relation, Bokhris et al. announced that oil extract of Siberian pine cone can be used as a source of natural antioxidants and antimicrobial compounds (3).

Therefore, the extract of pine cone which contains antioxidants such as Thymoquinone, Terpenes, Tanni, etc. (2,

5, 24, 25, 32, 33) causes a reduction in urinary excretion of ions through trapping the free radicals and inhibiting the formation of tubular damage. It also can lead to an increase in creatinine clearance and a reduction of urea and nitrogen in plasma. Ethanol extract of *Cassioeridentalis*, too, has the same effect as Pine extract (35).

It should be noted that several plant extracts prevent nephrotoxicity induced by gentamicin, among them is *Nigella Sativa* (36). Moreover, vitamins E and C cause a decrease in gentamicin-induced renal toxicity and the combined administration of the drugs has greater effects (37).

Aged garlic extract or AgE, as a natural antioxidant, plays a protective role against gentamicin-induced nephrotoxicity (9).

Vitex extract contains natural antioxidant that prevents kidney damage in rats (38).

Hydraulic extract of ginger has showed protective effect on kidney cells of rats poisoned by lead which is related to its phenolic and ethonolic compounds leading to neutralizing free radicals and inducing the repair of renal cells through its antioxidant properties (39).

Kelussiaodoratissima showed protective effect on kidney while *Cichoriumintybus* did not have such effect. The extract from *Capparisspinosa*, particularly in higher doses, showed poisoning effects on kidney (40). Utilizing the extract from carrot seed decreases Lipid Peroxidation, and controls gentamicin- induced oxidative stress through increasing antioxidant levels in blood and enhancing the rate of para-oxidation activity (41).

In summary, the present study showed that the aqueous extract of Siberian pine cone can improve and prevent gentamicine-induced kidney toxicity through its antioxidant compounds.

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