Original Article

Effect of High Doses of Salep Aqueous Extract on Serum Levels of Urea Nitrogen, Creatinine, Uric Acid, and Kidney Histopathological Changes in Adult Male Wistar Rats

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ABSTRACT

Kidneys are critical in the clearance and maintenance of active metabolites. One of the medical properties of Salep is treating bladder and kidney inflammation. Due to the widespread use of Salep in traditional medicine and the food industry, and since the effects of Salep on kidney function have not been studied, the present study aimed to investigate the impact of Salep on kidney function. In this experimental study, 48 male rats were divided randomly into six groups as control, sham, and four experimental groups receiving different doses of Salep intraperitoneally (80, 160, 320, and 640 mg/kg). On day 29, after weighing the animals, blood samples were taken from the heart, and serum blood urea nitrogen (BUN), uric acid, and creatinine were analyzed and compared in different groups. All the animal's kidneys were exposed after dissection, and tissue sections were prepared for histopathological evaluation. From day 28 to 29, rats were kept in metabolic cages to collect urine samples and measure water intake and urine volume. The serum concentration of BUN and uric acid in the groups receiving Salep at all doses decreased non-significantly compared to the control group. Furthermore, a significant reduction was seen in creatinine serum levels in groups receiving 320 and 640 mg/kg of Salep extract (P<0.05). No evidence of damage to renal tissue was observed in this study. In conclusion, Salep could decrease serum BUN, uric acid, and creatinine levels due to its antioxidant properties and had no devastating effect on kidneys.

Keywords: BUN, Creatinine, Kidney, Salep, Wistar rats

1. Introduction

Kidneys have a critical role in the clearance and maintenance of active metabolites and many metabolites are excreted through the kidneys. The results of previous research have demonstrated that decreased creatinine clearance, increased serum levels of blood urea nitrogen (BUN) and serum uric acid, and severe tissue damage are the main symptoms of kidney failure and kidney tubule injuries (1). Measurement of BUN and creatinine is considered a valid test for renal function evaluation (2).

Creatinine is a protein derived from muscle creatine. After dehydration of creatine in muscles and without catalysts, creatine is converted to creatinine and enters blood circulation. Creatinine mostly derives from skeletal muscles and excretes only through the kidneys; therefore, serum creatinine measurement indicates kidney function; accordingly, whenever renal function is impaired, serum creatinine level increases (3).

Blood urea nitrogen is important in the metabolism of nitrogen-containing compounds in animals and is considered the main nitrogen-containing substance in the urine of mammals. Urea is produced by the oxidation of amino acids or ammonia as part of the urea cycle. Urea is dissolved in the blood (2.1 to 8.5 mmol/L) and is excreted by the kidneys as a component of urine. Regulation of the urea level by the kidneys is a vital part of human body metabolism (3).

Uric acid is excreted primarily by the kidneys, with a small amount excreted in the feces. In renal failure, serum uric acid levels rise (3). Increased serum urea may be associated with glomerular vascular damage, glomerular hypertension, and reduced kidney perfusion (4, 5).

Salep is a perennial plant that reaches a height of 70 cm. This plant mostly grows in forests, wetlands, and mountainous areas of Europe, northern and western Asia, and North Africa (5). Salep (Lancibracteata (C.koch) Renz Dactylorhiza) is from the orchid family (Orchidaceae), which has different species worldwide. Salep roots are usually harvested in early summer and their medicinal properties are retained almost for two years (6). Salep contains quercetin, nitrogenous materials, ferolic acid, starch, protein, glucomannan, glucose, daucosterol, cirsilineol, and steroids (7, 8). This plant is used in traditional medicine as a healing agent in treating breast disorders, gastrointestinal disorders, tuberculosis, diarrhea, Parkinson's, cancer, fever, and impotence. Salep is used in food engineering to prepare ice cream, sweets, and drinks (9, 10).

Salep root has a hot and humid nature in ancient Iranian medicine. One of the medical properties of Salep is the treatment of bladder and kidney inflammation (nephritis) (6). The findings of previous studies have also demonstrated that Salep has hepatoprotective effects. Pourahmad et al. (2015) showed that aqueous extracts of Salep could reduce serum levels of malondialdehyde (MDA) and total oxidant capacity (TOC), increase total antioxidant capacity (TAC), and had no adverse effects on rat liver (11). Moreover, numerous compounds in salep roots, such as glucomannan and polyphenols, have potent antioxidant properties (7, 11).

Therefore, due to the presence of antioxidant compounds, including flavonoids, in Salep and the protective effects of antioxidants, this study aimed to evaluate the impact of different doses of Salep aqueous extract on serum levels of serum BUN, creatinine, uric acid, and histopathologic changes in kidneys of rats.

2. Materials and methods

2.1. Collection and extraction of salep

Salep roots were collected from Yasouj City, Iran, washed and dried in the laboratory, mixed with Ethanol 96% in 1 to 5 proportions, and combined for 24 h at room temperature, rendering a homogeneous mixture. Then, the uniform solution was filtered and dried for 48 h to get a solid extract without ethanol. The final dried section was dissolved in distilled water (11).

2.2. Experimental animals

Forty-eight adult Wistar rats (230-250 g) were obtained from the Animal House of Jahrom University of Medical Sciences, Jahrom, Iran. The animal house temperature was maintained at 22±2°C with a 12h light/dark cycle. All animals were kept for two weeks before the experiment and had free access to food and water. All ethical points regarding working with laboratory animals were considered in this research. Furthermore, all animals were transferred and kept in metabolic cages between days 28 and 29. On these two days, all animals were weighed, 24-hour water consumption and 24-hour urine volume were recorded, and urine specimens were collected for further analysis. This research was approved by Jahrom University of Medical Sciences with the ethical code of IR.JUMS.REC.1394.099 and all ethical aspects of working with animals were considered and were in compliance with the Guide for the Care and Use of Laboratory Animals.

2.3. Experimental design

Previous studies and a pilot study revealed that Salep was chosen for the current experiment at doses of 80, 160, 320, and 640 mg/kg (6, 11). The rats were divided systematically randomly into six groups (n=8 each), as follows:

Control group: Rats did not receive any substance during the experiment.

Sham group: Rats were given distilled water intraperitoneally according to their body weight during the investigation.

Experimental groups 1, 2, 3, and 4: Rats were given daily Salep at 80, 160, 320, and 640 mg/kg/BW, respectively.

Salep aqueous extract was injected intraperitoneally daily for 28 days in all four groups.

2.4. Blood sampling

At the end of the study (day 29), after weighing the animals, blood samples were taken directly from their hearts by using 5 cc syringes (rats were anesthetized by ketamine and xylazine), and blood serum was collected after centrifugation (15 min, 3,000 RPM) and stored at -20°C until they were tested. Biochemical measurement kits (Pars Azmoon Company, Iran) using the colorimetric method and an autoanalyzer machine (Selectera XL model made in Holland) were used to assess BUN, uric acid, and creatinine.

2.5. Histological examination

After drawing the blood, for histological examination, both kidneys were separated, weighted precisely by digital scale (model: AND Japan, with an accuracy of 0.001), and fixed by 10% formalin. Kidney sections with a thickness of 5µm were prepared, stained with hematoxylin and eosin, and their histological and pathological changes were studied using a light microscope. Moreover, the kidney parameters were measured by Dinocapture software.

2.6. Statistical analysis

All values were given as mean±SD. Statistical analysis was carried out in SPSS21 software using a one-way analysis of variance (ANOVA) followed by the Duncan post hoc test. A statistical P-value of less than 0.05 was considered significant.

3. Results

3.1. Biochemical measurement

Based on the results of the current study, the mean serum level of BUN in groups receiving Salep extract at doses of 80 and 160 mg/kg decreased insignificantly compared to control and sham groups (Table 1). Measurement of serum BUN level in the group receiving Salep extract at a dose of 320 mg/kg showed a non-significant reduction of the BUN level compared to the control group and a significant decrease of this factor compared to the sham group ($P \leq 0.05$) (Table 1). The mean serum BUN level significantly decreased by 640 mg/kg Salep extract compared to the control and sham groups (Table 1). These results showed that Salep at the dose of 640 mg/kg had more effect on reducing serum BUN than other doses (Table 1).

Table 1. Serum and biochemical urine measurement in different groups (Mean±SD)

Parameters / Group	Control	Sham	Salep Extract 80 mg/kg	Salep Extract 160 mg/kg	Salep Extract 320 mg/kg	Salep Extract 640 mg/kg
First day body weight (g)	238.125±5.817 ns	240.125±5.166 ns	241.375±4.501 ns	239.125±5.356 ns	238.5±3.464 ns	240.625±4.718 ns
Twenty-ninth day body weight (g)	255.125±7.239 ns	257.375±5.370 ns	257.5±5.928 ns	257.625±5.449 ns	255.125±4.356 ns	259.125±4.673 ns
Change of body weight (g)	17.000±3.422 ns	17.250±1.281 ns	16.125±2.799 ns	18.500±2.878 ns	16.625±2.199 ns	18.500±3.251 ns
Weight of left Kidney (g)	0.924±0.083 ns	0.922±0.056 ns	0.926±0.077 ns	0.922±0.094 ns	0.951±0.075 ns	0.948±0.080 ns
Weight of right Kidney (g)	0.968±0.083 ns	0.958±0.073 ns	0.922±0.069 ns	0.973±0.048 ns	0.925±0.047 ns	0.963±0.075 ns
Weight of kidneys (g)	1.892±0.154 ns	1.880±0.086 ns	1.848±0.113 ns	1.896±0.071 ns	1.876±0.110 ns	1.911±0.112 ns
Water consumption (ml/day)	19.625±2.559 a	20.125±2.587 a	21.250±2.314 a	22.000±2.267 ab	23.750±2.052 bc	25.125±2.031 c
Volume of urine (ml/day)	9.225±1.025 a	9.375±1.775 a	9.350±1.383 a	10.312±1.565 ab	10.600±0.866 ab	11.137±0.871 b
BUN serum (mg/dl)	1.728±0.236 bc	1.885±0.241 c	1.742±0.250 bc	1.700±0.223 bc	1.614±0.157 B	1.214±0.158 a
UA serum (mg/dl)	22.857±2.853 b	23.000±2.160 b	21.856±3.976 ab	20.428±2.699 ab	20.142±3.078 ab	18.858±2.544 a
UA urine (mg/dl)	0.425±0.070 a	0.412±0.135 a	0.462±0.091 ab	0.475±0.103 ab	0.487±0.136 Ab	0.575±0.104 b
Cr serum (mg/dl)	0.635±0.036 c	0.627±0.034 c	0.611±0.038 bc	0.582±0.033 ab	0.554±0.029 A	0.552±0.048 a
Cr urine (mg/dl)	36.375±2.326 a	35.812±4.240 a	36.462±4.385 a	38.900±2.939 ab	41.637±4.260 Bc	43.100±3.043 c
Ca urine (mg/dl)	5.362±1.063 ns	5.287±0.864 ns	4.975±0.728 ns	4.987±0.622 ns	5.075±0.654 Ns	4.850±0.618 ns

- According to the Duncan test, means with at least one letter in common in each row have no significant difference.
- The means are presented in the form of Mean ±SD.
- $P < 0.05$ is considered statistically significant.

BUN: Blood Nitrogen Urea, UA: Uric acid, Cr: Creatinine, Ca: Calcium, ns: non-significant, mg: milligram, dl: deciliter, g: gram, kg: kilogram

The mean serum level of uric acid in groups receiving Salep extract at doses of 80, 160, and 320 mg/kg decreased insignificantly compared to the control and sham groups. However, a significant decrease was observed in serum uric acid level in the group receiving 640 mg/kg of Salep extract compared to the control and sham groups ($P \leq 0.05$) (Table 1). These results also demonstrated that Salep at a 640 mg/kg dose reduced serum uric acid more than other doses (Table 1).

The mean serum level of creatinine in the group receiving Salep extract at a dose of 80 mg/kg decreased insignificantly compared to the control and sham groups; however, a significant decrease was

seen in serum creatinine levels in groups receiving 160, 320, and 640 mg/kg of Salep extract compared to control and sham groups ($P \leq 0.05$) (Table 1). These results indicated that Salep at 320 and 640 mg/kg doses had more subtractive effects on serum creatinine than other doses (Table 1).

3.2. Histopathological and histomorphometry examination

On microscopic examination of renal tissues of all four experimental groups, neither trace of congestion, venous distention, tubular necrosis, inflammatory cell infiltration, or glomerular changes was observed nor significant structural changes in renal tissues (Table 2). The diameter of proximal and distal tubules, the loop of henna, collecting tubule, glomerulus, Bowman's capsule, urinary space, cortical thickness, and kidney modulus did not change significantly between the experimental and control and sham groups (Figure 1).

Table 2. Renal tissue structure analysis in different groups (Mean±SD)

Parameters / Group	Control	Sham	Salep Extract 80 mg/kg	Salep Extract 160 mg/kg	Salep Extract 320 mg/kg	Salep Extract 640 mg/kg
CT (mm)	0.1263±0.1437 ns	0.095±0.0121 ns	0.1017±0.0137 ns	0.0938±0.0152 ns	0.0983±0.0146 ns	0.0921±0.0128 ns
LH (mm)	0.0338±0.0071 ns	0.0350±0.0059 ns	0.0346±0.0078 ns	0.0342±0.0077 ns	0.0454±0.0568 ns	0.0333±0.0056 ns
DT (mm)	0.0725 ±0.0079 ns	0.0713±0.0068 ns	0.0714±0.0085 ns	0.0721±0.0078 ns	0.0733±0.0081 ns	0.0734±0.0063 ns
PT (mm)	0.1071±0.0142 ns	0.1075±0.1032 ns	0.1054±0.0144 ns	0.0992±0.0124 ns	0.1054±0.0141 ns	0.1021±0.0135 ns
US (mm)	0.0375±0.0562 ns	0.0308±0.0077 ns	0.0306±0.0068 ns	0.0313±0.0084 ns	0.0275±0.0071 ns	0.0258±0.0072 ns
Gl (mm)	0.2479±0.0223 ns	0.2525±0.0287 ns	0.2483±0.0265 ns	0.2479±0.0245 ns	0.2454±0.0309 ns	0.2396±0.0244 ns
BC (mm)	0.2550±0.0230 ns	0.2592±0.0216 ns	0.2508±0.0273 ns	0.2613±0.0275 ns	0.2654±0.0257 ns	0.2654±0.0285 ns
M Tick (cm)	1.0042±0.0919 ns	0.9867±0.0737 ns	0.9725±0.0471 ns	0.9829±0.0553 ns	0.9717±0.0507 ns	0.9892±0.0527 ns
C Tick (cm)	0.5008±0.0320 ns	0.5071±0.0332 ns	0.4983±0.0431 ns	0.4963±0.0567 ns	0.4858±0.0446 Ns	0.4963±0.0605 ns

CT: Collecting Tubule, LH: Loop of Henle, DT: Distal Tubule, PT: Proximal Tubule, US: Urinary Space, Gl: Glomeruli, BC: Bowman's capsule, M Tick: Medullary Thickness, CT: Cortex Thickness, g: gram, kg: kilogram, ns: non-significant, mm: millimeter, cm: centimeter

4. Discussion

Decreased creatinine clearance, increased serum BUN and Uric acid, and severe kidney tissue damage are the main symptoms of kidney tubule injuries and subsequent kidney failure (1). The results of the present study showed that the mean concentration of BUN and uric acid in the group that received the 640 mg/kg dose of Salep significantly decreased compared to the control group. Furthermore, the mean serum creatinine level at doses of 320 and 640 mg/kg reduced significantly compared to the control group.

Salep is a valuable source of glucomannan, with a percentage of 7% to 61% in different species (9). Glucomannan is a water-soluble fiber that effectively controls weight loss, blood glucose, and cholesterol (12-14). Zhang et al. (2016) noted that glucomannan could reduce the serum levels of BUN, uric acid, and creatinine, while also lowering the activity of xanthine oxidase (XOD) and adenosine deaminase (ADA) enzymes in hyperuricemia rats. The XOD and ADA enzymes play a crucial role in uric acid synthesis. The ADA enzyme induces the deamination of adenosine and converts it into inosine, which turns into xanthine and uric acid by the XOD enzyme. Therefore,

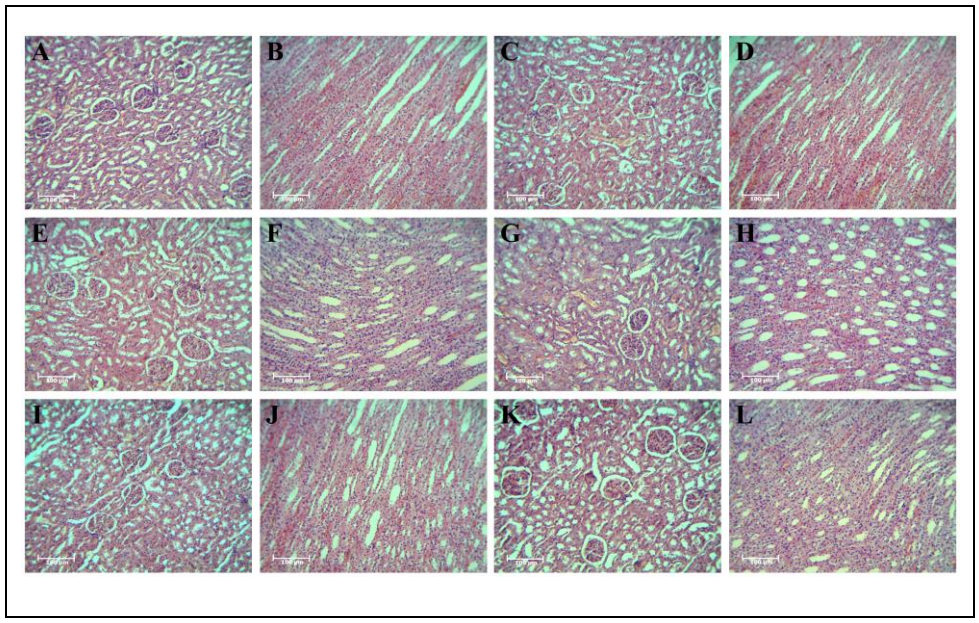

Figure 1. Microscopic View of the Kidney Tissue in Studied Groups

glucomannan could decrease serum BUN, uric acid, and creatinine by reducing and influencing the activity of XOD and ADA enzymes in hyperuricemia conditions (15). In another study, glucomannan treatment significantly decreased glycosuria, ketonuria, and proteinuria. It improved the urea cycle and metabolism of lipids, glucose, and amino acids in diabetic rats, thus effectively managing diabetic kidney disease (16). The results of a study by Chiavaroli and Colleagues investigating the effects of nutritional fiber on chronic renal failure (CKD) in 2014 demonstrated that healthy fibers reduced urea and creatinine serum levels in CKD patients (17). Likewise, different studies have shown the effect of dietary fiber supplementation on reducing serum urea, creatinine, and uremic toxins in CKD patients (17-19). Flavonoids, such as quercetin, are among the compounds found in Salep and possess inhibitory properties on XOD enzymes (20). MO et al. (2007) studied the hypouricemic properties of various flavonoids in rats and reported that the hypouricemic properties of flavonoid compounds were directly related to the inhibition of the XOD enzyme (21). Heidari et al. (2009 and 2010) showed that phenolic compounds, such as quercetin, hesperetin, and camphorol, found in oranges and parsley could competitively inhibit XOD enzyme and thus reduce uric acid production (22, 23). Studies on the effects of different doses of Salep extract or Salep compounds on kidney tissue are consistent with the biochemical findings of the current study (15-19). Different doses of Salep extract did not cause any change in renal tissue, such as hyperemia, vein dilatation, tubular necrosis, inflammatory cell infiltration, and glomerular changes, compared to the control group. Therefore, based on the results of this study, Salep root extract had no degenerative effects on the kidney. This might be due to the presence of antioxidant compounds in Salep extract. Antioxidants play a very important role in protecting the internal body organs against damage caused by free radicals (3). The results of a study by Pourahmad et al. (2015) on biochemical parameters and liver tissue also showed that the aqueous extract of Salep root increased the TAC, decreased the lipid peroxidation indexes, such as MDA and TOC levels in rats, and had a protective effect on the liver tissue (11). Salep extract components, including glucomannan and flavonoids, have significant antioxidant properties. Studies have demonstrated that glucomannan can inhibit oxidative stress (24, 25). Furthermore, glucomannan can increase the level of superoxide dismutase and catalase, which are endogenous antioxidant enzymes, and reduce malondialdehyde levels (26). Polyphenol compounds and flavonoids, such as quercetin, can also protect cells against the depletion of reduced glutathione by increasing the antioxidant enzyme

capacity (glutathione, glutathione reductase, glutathione peroxide, and catalase) (27). The antioxidant activity of phenolic compounds is essentially due to their reducing properties, which allow them to act as a reducing agent, a hydrogen supply, and an inactivator of oxygen radicals (28). The presence of adjacent hydroxyl (OH) groups also increases the antioxidant properties of quercetin (29-31). Ferulic acid, one of the Salep compounds, is a powerful antioxidant for clearing free radicals (32, 33). Ferulic acid is a phenolic compound with a protective role in the kidney in nephrotoxicity models. The results of a study by Salma Mukhtar et al. in 2018 showed that ferulic acid could improve the increase in BUN and creatinine levels, the reduction of antioxidant defense, and the release of inflammatory cytokines in mice balb/c with kidney damage caused by lipopolysaccharide. Furthermore, ferulic acid showed a protective function against fibrosis caused by sepsis and kidney damage. This study suggested that ferulic acid had strong nephroprotective effects (33). In addition, Zhou et al. (2018) reported that ferulic acid significantly weakened renal damage in renal I/R-operated mice, as shown by decreasing levels of serum creatine and blood urea nitrogen, recovering renal histopathological changes, and cell apoptosis. They demonstrated that ferulic acid secured against kidney I/R damage by decreasing inflammation and apoptosis (32). Daucosterol is another compound found in Salep, a natural phytosterol that exerts its biological activity in many diseases and has numerous effects, such as relieving inflammation and oxidative stress (34-36). It has also been determined that the oil extract of pomegranate seeds, having antioxidant components, such as daucosterol, can reduce serum creatinine, blood urea nitrogen, glucose, and urine protein in the nephrotoxicity mouse model (37). In another study conducted by Zhang et al. (2023), it was found that daukosterol could reduce oxidative stress, increase antioxidant factors, and improve liver fibrosis in rats with alcoholic fatty liver (38). Moreover, Jang J et al. reported that daukosterol decreased reactive oxygen species (ROS) induced by dextran sodium sulfate (39). Considering the antibacterial effects of Salep, Akkaya et al. (2020) reported that this compound could effectively control creatinine, urea, and uric acid levels in acute glomerulonephritis (40). In addition, Usta et al. (2017) showed that Salep had a probiotic effect and caused the growth of bifidobacteria in the digestive system. These bacteria have a direct role in the metabolism of amino acids and the production and excretion of urea from the body; as a result, Salep indirectly affects the level and clearance of blood urea (41). In expansion, flavonoids and antioxidants can diminish creatinine levels and normal kidney tissue damage (42). Daucosterol is a natural steroid found in various plant sources, such as carrots, seaweed, ginseng, and Salep (43). There is limited research on the specific effects of daucosterol on the kidney and renal system. However, some studies suggest that daucosterol may have potentially beneficial effects on the kidney and renal system. Zhang et al. (2017) found that daucosterol extracted from carrots had antioxidant and anti-inflammatory effects *in vitro*. These effects may help to protect the kidneys from oxidative stress and inflammation, which are known as contributors to the development and progression of kidney disease (44). Another study published in the Journal of Agricultural and Food Chemistry reported that daucosterol extracted from sea buckthorn berries had a protective effect against cisplatin (chemotherapy agent with nephrotoxicity) and induced kidney injury in rats. Cisplatin is a commonly used chemotherapy drug that can cause kidney damage as a side effect (45). In a study by Rajavel et al. in 2017, it was found that daukosterol alone or in combination with beta-sitosterol did not change kidney tissue and serum creatinine and urea levels (46). Another chemical agent found in Salep is cirsilineol, which belongs to a class of chemicals called flavones, which are a type of flavonoid (47). Several studies have investigated the potential renal protective effects of flavones and related chemicals in animal models (48-51). Gong (2021) found that the oral organization of cirsilineol on hydrochloric acid/ethanol-induced gastric injury in rats occurred in a critical change of kidney function parameters (urea, creatinine, total protein, albumin, and globulin). Additionally, advancement within the kidney parameter showed a non-toxic profile of cirsilineol, clinically (52). While there is no direct evidence of the potential effect of cirsilineol on the kidney and renal system, the fact that other chemicals from the same family have shown potential renal protective effects in animal studies suggests that cirsilineol

may have similar effects. However, further research is needed to confirm this hypothesis. According to the results of this study, it was revealed that Salep could reduce serum levels of BUN, uric acid, and creatinine and had no harmful effect on renal tissue, thus suggesting their non-toxic role. The progression of tissue damage likely depends on the adjustment between the era of ROS and the tissue antioxidant defense mechanism. These effects are mainly due to its antioxidant properties. Additionally, it has a protective impact on the liver and digestive system. Therefore, it is suggested that further studies investigate the specific effects of this plant on renal failure models. It is also recommended that a detailed comparison be conducted between the very different species of this plant in Iran.

Authors' Contribution

Study concept and design: H.K.J., S.A., H.A., and J.S.
Acquisition of data: H.K.J., A.M., M.A.B., and N.S.J.
Analysis and interpretation of data: H.K.J, S.A, H.A., and A.M.
Drafting of the manuscript: H.K.J., A.M., and N.S.J. Critical revision of the manuscript for important intellectual content: S.A., H.A., N.S.J., M.A.B., J.S., A.M., and H.K.J. Statistical analysis: H.K.J., and A.M. Administrative, technical, and material support: H.K.J

Ethics

The student research committee of Jahrom University of medical sciences with the ethical code of IR has approved this study.JUMS.REC.1394.099

Conflict of Interest

To the best of our knowledge, no conflict of interest exists.

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