

## Research Article



## Protective Effect of Flavonoid Rich Fraction of *Sesbania grandiflora* Leaves against Chemically Induced Urolithiasis *in vitro* and *in vivo*

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Received: 20-11-2021; Revised: 25-01-2022; Accepted: 03-02-2022; Published on: 15-02-2022.

### ABSTRACT

The present study is to evaluate the antiurolithiatic effect of flavonoid rich fraction of *Sesbania grandiflora* leaves against ethylene glycol and ammonium chloride induced urolithiasis in experimental animals. Urolithiasis was induced in rats by feeding them with ethylene glycol and ammonium chloride in drinking water. Antiurolithiatic activity of *S. grandiflora* was evaluated at two doses (200, 400 mg/kg) in experimental animals. Cystone (750mg/kg) was used as standard drug. The dose effect was estimated by biochemical changes in urine, serum and histological changes in kidney. Ethylene glycol-ammonium chloride feeding caused an increase in urinary volume, oxalate, phosphate, urea, uric acid levels along with decrease in magnesium. Treatment with *S. grandiflora* prevent the elevation of serum creatinine, uric acid, urea and blood urea nitrogen levels as well as associated biochemical changes in animals. Histological study revealed minimum damage and less number of calcium oxalate deposits in the kidney of *S. grandiflora* treated animals. These results indicate that *S. grandiflora* reduced as well as prevented the growth of urinary stones. These finding supports the traditional use of *S. grandiflora* for urolithiasis.

**Keywords:** Urolithiasis, Calcium Oxalate, *Sesbania grandiflora*, Ethylene glycol, Ammonium chloride.

### QUICK RESPONSE CODE →

#### DOI:

10.47583/ijpsrr.2022.v72i02.020



DOI link: <http://dx.doi.org/10.47583/ijpsrr.2022.v72i02.020>

### INTRODUCTION

Urolithiasis means origination of stones anywhere in the urinary tract including kidney, ureter and urinary bladder. The supersaturation of urine with stone-forming salts leads to urolithiasis. <sup>1</sup> Amongst many types of stone, the most common were calcium oxalate. Stone formation occurs with several physicochemical events such as begins with crystal nucleation, growth, aggregation which retain within the urinary tract. It is a worldwide medical problem that occurs about 12% of the population, three times more likely to develop in men than in women. <sup>2</sup> If urinary calculi left untreated it can lead to obstruction, hydronephrosis, hemorrhage, and infection in the urinary tract system. <sup>3</sup> Surgical operation, lithotripsy, local calculus disruption are the modern medicine methods in which high power laser was used to remove calculi. Acute renal injury decreased renal function and increased stone recurrence were serious complications of these methods. <sup>4</sup> The recurrence rate of urolithiasis is around 10% in 1 year, 33% in 5 years and 50% in 10 years. <sup>5</sup> However, other surgical treatments remain costly, and in most cases shows side effects. Therefore, it is worth to look for an alternative to those conventional treatments, such as the use of medicinal plants. In the indigenous

systems of medicine, medicinal plants have an alternative source for new drugs, many plants have claimed to cure urinary stones.

*Sesbania grandiflora* (Family: Leguminosae) commonly known as 'Humming bird', a well-known medicinal plant of the Indian medicinal system, has been used to treat a variety of diseases. The major chemical constituents of *S. grandiflora* leaves are flavonoids, aspartic acid, oleanolic acid, glucuronic acid, linolenic acid, amino acid, kaempferol. The leaves of *S. grandiflora* have been reported to have various pharmacological activities such as anxiolytic, hepatoprotective, antioxidant, diuretic and nephroprotective. Based on the literature as *S. grandiflora* having diuretic & nephroprotective activity hence we postulated that *S. grandiflora* might be effective in urolithiasis. Therefore, in the present study, we investigated the effectiveness of flavonoid-rich fraction of *S. grandiflora* on calcium oxalate crystallization in-vitro and ethylene glycol, ammonium chloride induced renal calculi in-vivo in Wistar rats.

### MATERIAL AND METHODS

#### Collection and authentication of plant material

The leaves of *Sesbania grandiflora* Linn were collected in the month of September 2019 from local market of District Pune, Maharashtra, India. The plant was identified and authenticated by Department of Botany, Savitribai Phule Pune University, Pune.

#### Extraction of plant material and preparation of fraction

Leaves of *S. grandiflora* were dried, powdered and then preserved in an air-tight container. The dried powder (450



g) were macerated by soaking in 1500 ml of ethanol for ten days with occasional shaking. The extract then filtered off and dried at room temperature to prevent loss of important plant constituents then to obtain a crude ethanolic extract of *S. grandiflora* (yield-10g). The ethanolic extract then fractionated with ethyl acetate and used for the current investigation.

#### Experimental animal

Healthy male Wistar rats (120-170g) were procured from Global Bioresearch Solution Pvt Ltd, Nhavi, Tal. Bhor, Dist. Pune (India). Animals were housed in a group of 6 per cage in standard polypropylene cages (32.5x21x14) cm lined with raw husk. The animal house was maintained on 12 light/dark cycle approximately 22±2°C, relative humidity 60-70%. All animals were provided with a standard laboratory diet (Nutrivet Life science, Maharashtra, India) and water ad libitum. All experimental procedures were carried out by the guidelines prescribed by CPCSEA and study was approved by the IAEC. (884/PO/Re/S/05/CPCSEA)

#### In-vitro activity

##### Nucleation assay

Nucleation assay was used to determine inhibitory effect of *S. grandiflora* on calcium oxalate crystals by spectrometric method. In this study, crystallization was initiated by adding calcium chloride (5 mmol/L) and sodium oxalate (7.5 mmol/L) solutions, which were prepared in a Tris Buffer (0.05 mol/L) and sodium chloride (0.15 mol/L) at pH 6.5. 1ml of extract solution (200mg/kg, 400mg/kg) was mixed with 2ml calcium chloride solution followed by addition of sodium oxalate solution. The rate of nucleation of crystals was determined by comparing the induction time of crystals in the presence of the extract and without extract. The absorbance (optical density, OD) was recorded at 620 nm in UV visible spectrophotometer. The percentage inhibition was calculated as using under mentioned formula. <sup>6</sup>

$$\% \text{ inhibition} = 1 - \left( \frac{\text{turbidity (sample)}}{\text{turbidity (Control)}} \right) \times 100$$

##### Aggregation assay

The calcium oxalate monohydrate crystals were prepared by mixing solutions of sodium oxalate and calcium chloride at 50 mmol/L, heated to 60°C in a water bath for one hour and then incubated overnight at 37°C. The calcium oxalate crystals were dissolved in Tris buffer (0.05 mol/L) and sodium chloride (0.15 mol/L) at pH 6.5. 1ml of extract solution (200mg/kg, 400mg/kg) was mixed with 2ml calcium oxalate solution and absorbance was recorded at 620nm at interval of 0, 30, 60, 90 min. The percent inhibition of aggregation was estimated as described in the nucleation assay. <sup>6</sup>

#### In-vivo activity

Ethylene glycol and ammonium chloride induced hyperoxaluria model was used to induced urolithiasis in Wistar rats. Two doses of *S. grandiflora* extract were used to evaluate antiurolithiatic activity. Animals were assigned into five groups each containing six animals each. Group I served as normal control which received standard rat food and drinking water *ad libitum*. All the remaining groups received calculi inducing treatment for 28 days which contains 0.75% v/v ethylene glycol with 1% w/v ammonium chloride in drinking water. Group II served as diseased control which received calculi inducing treatment. Group III served as standard treatment group which received cystone (750 mg/kg) from 15<sup>th</sup> to 28<sup>th</sup> day. Group IV and V served as treatment groups which received *S. grandiflora* extract (200mg/kg and 400mg/kg) from 15<sup>th</sup> to 28<sup>th</sup> day. <sup>7</sup>

#### Collection and analysis of urine

After 28 days of calculi inducing treatment, all animals were kept in individual metabolic cages for 24 hours and urine samples were collected. The collected urine samples were analyzed for uric acid, urea, creatinine, calcium, oxalate, magnesium and phosphate. The urine samples were used for the study of crystalluria to estimate the presence of calcium oxalate crystals by observing under microscope.

#### Collection and analysis of blood

After 28<sup>th</sup> day, blood was collected from retro-orbital plexus under light anesthesia. Serum were separated and analysed for uric acid, urea, creatinine, blood urea nitrogen (BUN).

#### Histopathology

After collection of blood and urine, animals were sacrificed, both kidneys were identified, carefully dissected and preserved in specimen container containing 10% formalin and histopathological examination were done. The histological changes such as necrosis, hemorrhages, congestion, and swelling were observed as a damage index.

#### Statistical analysis

The data were analysed using Graph pad prism software. The results were analysed by one way ANOVA followed by Tukey's Multiple Comparison test. All results were expressed as mean ± standard error of mean (SEM). Differences between groups were considered significant at  $p < 0.05$ .

## RESULTS

#### In-vitro activity

##### Nucleation assay

Supersaturation of urine leads to crystallization of calcium oxalate crystals within urinary tract. Nucleation is the prior step for the initiation of crystals. The crystal growth



occurred and form agglomeration, this process is called as crystal aggregation. In current study Citric acid and Hydrochloric acid were taken as standard. It was observed that *S. grandiflora* (200mg/kg and 400mg/kg) inhibited crystallization by preventing nucleation of calcium oxalate and disintegrate into smaller particles. (Fig. 1)

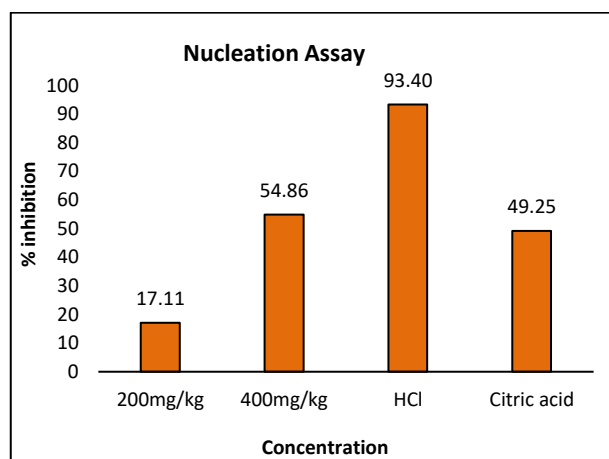
### Aggregation assay

*S. grandiflora* were compared with citric acid and hydrochloric acid which showed significant inhibition of aggregation of calcium oxalate crystals at 0, 30, 60 and 90 min at absorbance 620nm and prevented the development of calcium oxalate crystals. (Fig. 2)

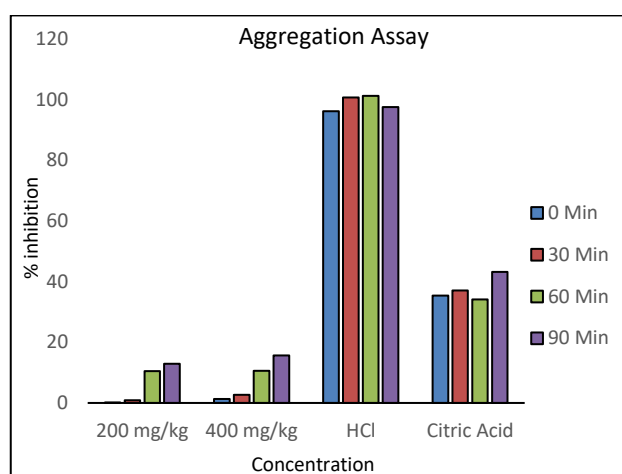
### In-vivo activity

#### Effect of *S. grandiflora* on urine analysis

The urinary excretion of various urolithiatic promoters such as calcium, oxalate, magnesium, phosphate, uric acid, urea, and creatinine were measured. There was significant increase in the urinary excretion of urolithiatic promoters in the urine of diseased control rats as compared to normal control rats. However, administration with *S. grandiflora* 200mg/kg and 400mg/kg (Table1) showed a significant reduction in urinary excretion of calcium, oxalate, phosphate, uric acid, urea, creatinine as compared to urolithiatic control group rats. The level of magnesium decreases in urolithiatic animals compared to normal control. Magnesium is the inhibitor of calcium oxalate crystal. After treatment with *S. grandiflora* at both the doses (200mg/kg, 400mg/kg) showed significant increase in the level of magnesium. Higher dose of *S. grandiflora* (400mg/kg) showed more reduction in urolithiatic promoters as well as increase in the level of magnesium which act as an inhibitor of calcium oxalate crystals. These results were also compared with the standard drug, Cystone also showed significant reduction in urolithiatic promoters and also increases the level of magnesium.



**Figure 1:** Nucleation assay shows, extract of *S. grandiflora* (200mg/kg and 400mg/kg) against citric acid and hydrochloric acid at absorbance 620nm.



**Figure 2:** Aggregation assay shows, extract of *S. grandiflora* (200mg/kg and 400mg/kg) against citric acid and hydrochloric acid at absorbance 620nm.

**Table 1:** Effect of *S. grandiflora* on urine analysis

Groups	Calcium	Oxalate	Magnesium	Phosphate	Uric acid	Urea	Creatinine
Normal control	0.122 ±0.009	2.825 ±0.236	30.328 ±0.546	2.997 ±0.172	4.302 ±0.303	20.707 ±0.396	27.675 ±1.331
Disease control	1.990 ±0.062	8.895 ±0.272	18.720 ±0.467	8.285 ±0.277	8.708 ±0.383	27.070 ±0.441	32.395 ±1.046
Standard control	1.728 ±0.086 <sup>†</sup>	7.930 ±0.213 <sup>†</sup>	21.717 ±0.334 <sup>#</sup>	7.765 ±0.221	7.885 ±0.320	25.917 ±0.436	30.920 ±1.246
<i>S. grandiflora</i> (200mg/kg)	0.972 ±0.045 <sup>*</sup>	7.365 ±0.173 <sup>*</sup>	23.538 ±0.375 <sup>*</sup>	7.257 ±0.165 <sup>†</sup>	6.647 ±0.374 <sup>#</sup>	24.788 ±0.462 <sup>#</sup>	30.192 ±1.239
<i>S. grandiflora</i> (400mg/kg)	0.643 ±0.071 <sup>*</sup>	5.647 ±0.227 <sup>*</sup>	26.393± 0.636 <sup>*</sup>	5.675 ±0.212 <sup>*</sup>	5.938 ±0.323 <sup>*</sup>	23.660 ±0.444 <sup>*</sup>	29.347 ±1.492

Values are expressed as mean ± SEM; n = 6; Data analyzed by One-way ANOVA test followed by Tukey's multiple test for comparison. Level of significance †P < 0.05; #P < 0.01; \*P < 0.001. Values of Disease control group were compared with Normal control group those of Standard group and *S. grandiflora* (200mg/kg, 400mg/kg) with Disease control group.

### Effect of *S. grandiflora* on serum analysis

Urolithiasis induction caused impairment of renal functions which increased serum markers such as uric acid, urea, creatinine and blood urea nitrogen. On administration with *S. grandiflora* (200mg/kg and 400

mg/kg) showed significant reduction in the serum uric acid, urea, creatinine and blood urea nitrogen (Table2). Treatment with higher dose of *S. grandiflora* (400mg/kg) and cystone showed reduction in serum markers as compared to disease control.

**Table 2:** Effect of *S. grandiflora* on serum analysis

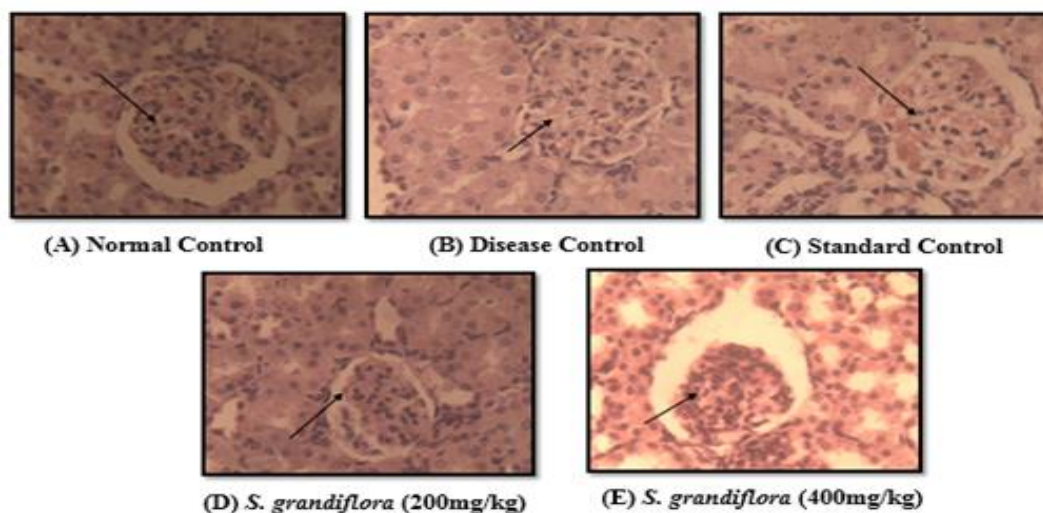
Groups	Uric acid	Urea	Creatinine	Blood urea nitrogen
Normal control	3.413±0.194	41.538±0.616	0.373±0.040	9.038±0.426
Disease control	8.978±0.245	50.583±0.894	1.190±0.092	20.038±0.722
Standard control	8.038±0.180 <sup>†</sup>	46.150±0.532 <sup>#</sup>	0.997±0.083	18.603±0.859
<i>S. grandiflora</i> (200mg/kg)	7.263±0.127 <sup>*</sup>	43.250±0.736 <sup>*</sup>	0.812±0.051 <sup>#</sup>	18.197±0.851
<i>S. grandiflora</i> (400mg/kg)	5.900±0.224 <sup>*</sup>	40.600±0.619 <sup>*</sup>	0.590±0.051 <sup>*</sup>	16.003±0.514 <sup>#</sup>

Values are expressed as mean± SEM; n = 6; Data analyzed by One-way ANOVA test followed by Tukey's multiple test for comparison. Level of significance <sup>†</sup>P < 0.05; <sup>#</sup>P < 0.01; <sup>\*</sup>P < 0.001. Values of Disease control group were compared with Normal control group those of Standard group and *S. grandiflora* (200mg/kg, 400mg/kg) with Disease control group.

### Histopathological study of Kidney

Histopathology reports showed normal structure of kidney tissue in normal control animals. Kidney structure of calculi-induced animals showed marked histological changes such as increased in the damage index with increased in necrosis, dilatation of tubules along with interstitial fibrosis. All these histological changes and

damage index were significantly reduced in animals treated with *S. grandiflora* (200mg/kg and 400mg/kg) and cystone. Deposition of calcium oxalate crystals were small and less abundant in kidneys of animals treated with *S. grandiflora* (200mg/kg, 400mg/kg) as compared to those in the calculi-induced kidneys. Higher dose of *S. grandiflora* (400mg/kg) showed more reduction in the damage index as well as in crystal deposition.



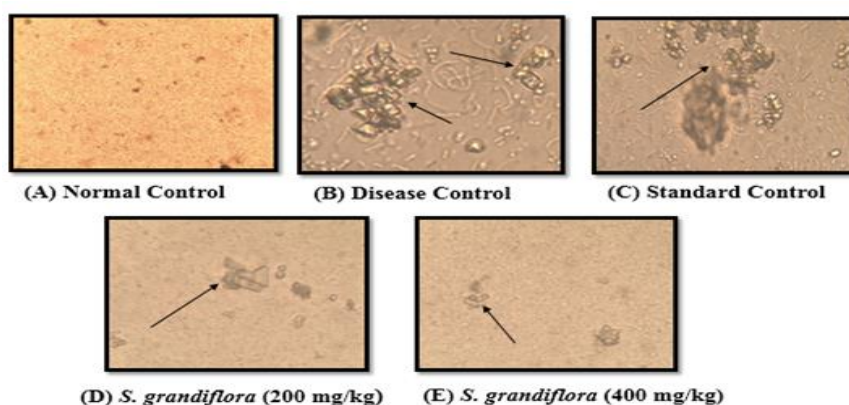
**Figure 3:** Microscopic images of kidney sections by trinocular microscope (100x). Normal control (A) showing intact structure with Bowman's capsule and blood vessels. Disease control (B) showing necrosis with congested blood vessels. Standard control (C) showing degenerative changes in tubular epithelial cells, dilated and congested blood vessels. *S. grandiflora* (200mg/kg) (D) showing normal cellularity with less congested blood vessels. *S. grandiflora* (400mg/kg) (E) showing normal cellularity, intact structure with Bowman's capsule and normal blood vessels.

### Microscopy of urine

Microscopic examination of urine (Fig. 4) showed calcium oxalate deposition in urolithiasis induced animals. Standard drug Cystone showed reduction in calcium oxalate crystals compared to urolithiatic control. Treatment with *S. grandiflora* (200mg/kg, 400mg/kg) prevented accumulation, deposition and supersaturation

of stone forming constituents, thereby inhibited formation of stones. *S. grandiflora* also dissolves stones by causing dissolution of mucin which is responsible to bind stone particles together. Furthermore, it has diuretic action that flushes out comparatively smaller stones from kidneys. Higher dose of *S. grandiflora* (400mg/kg) were more effective in reducing the risk of stone formation and decreases crystal size.





**Figure 4:** Microscopy of urine

## DISCUSSION

Supersaturation of urine along with stone forming agents is responsible for urolithiasis. In current study in-vitro crystallization and in-vivo experimental animal models by using Wistar rats have been used to study urolithiasis. In in-vitro crystallization, nucleation is an important step for the initiation of crystals, which then grows and form aggregates. The presences of acids such as aspartic acid, oleanolic acid, glucuronic acid, linolenic acid, amino acid present in the *S. grandiflora* were the main findings of the present study which inhibited nucleation of calcium oxalate in solution; with higher concentration (400mg/kg) of *S. grandiflora* extract smaller particles were observed. The result of the nucleation assay (Fig. 1) showed that the *S. grandiflora* contained nucleation preventing agents such as polyphenols, kaempferol, aspartic acid, oleanolic acid, glucuronic acid, linolenic acid and amino acid. In aggregation assay, particles may become large and occlude in the urinary tract. The result of the aggregation assay (Fig. 2) shows that *S. grandiflora* inhibited the agglomeration growth of calcium oxalate crystals.

In-vivo study is the most reliable method to induced urolithiasis in experimental animals by providing ethylene glycol and ammonium chloride in drinking water to rats. Therefore, we evaluated the antiurolithiatic activity of *S. grandiflora* on calcium oxalate urolithiasis using this model. To induce urolithiasis, male rats are selected because testosterone plays an important role in oxalate production which increases serum testosterone level in male rats thereby, liver increases endogenous production of oxalate and further low concentration of ethylene glycol and ammonium chloride solution induces calcium oxalate urolithiasis in male rats, similar results were not produce in females that is why male rats are more susceptible to develop calcium oxalate crystals than female rats. The biochemical mechanism of ethylene glycol and ammonium chloride induced urolithiasis are related to an increase in the urinary concentration of oxalate. Ethylene glycol absorbed in the intestine and oxidized to oxalic acid by non-specific dehydrogenase which leads to hyperoxaluria. Due to its poor solubility, oxalate precipitates in the urine as calcium oxalate and high level of oxalate and calcium

oxalate crystals especially damages epithelial cells in nephron which leads to nucleation followed by aggregation of crystals.<sup>8,9</sup> Ammonium chloride has been reported to accelerate urolithiasis.<sup>10</sup> After treatment for 28 days with urolithiasis inducing agents showed that urolithiatic animals excrete larger and aggregated stone particles as observed in urine. But after treatment with *S. grandiflora* extract for 14 days urinary crystals were reduced significantly at both low and high dose (200mg/kg, 400mg/kg). This could be helpful in reducing their chances of retention in the urinary tract. Treatment with *S. grandiflora* also increases urine volume but less than calculi-induced animals, due to diuretic effect of *S. grandiflora* which reduces calcium oxalate supersaturation in urine and thereby stone formation.<sup>11</sup>

The increased urinary calcium is responsible for favoring nucleation and precipitation of calcium oxalate in urine which further leads to crystal growth. In the present study, both the doses of *S. grandiflora* and Cystone showed reduction in urinary calcium as compared to disease control group. Reduction of calcium level in urine provides less calcium to bind with oxalate which leads to reduction of crystals, apparent in urinary microscopy observation. Urinary magnesium excretion was decreased in calculi-induced animals. Magnesium is the urinary inhibitor in crystallization. Low level of magnesium is responsible for stone formation. Magnesium form complex with oxalate and reduced the supersaturation of calcium oxalate by reducing the growth and nucleation of crystals. Treatment with *S. grandiflora* restored the magnesium level near to normal and reduced the growth of crystals. Higher dose (400mg/kg) of *S. grandiflora* extract shows elevation of magnesium level in urine. The result evidently indicates the efficacy of extract towards prevention of stone formation. An increase in urinary phosphate excretion along with oxalate stress provides suitable environment for stone formation by forming calcium phosphate crystals, which further induces deposition of calcium oxalate crystals. The *S. grandiflora* treatment showed reduction in the rate of urinary phosphate excretion and thereby reduces stone formation. An increase in nitrogenous substances in urine such as uric acid, urea and creatinine are the indicators for kidney and tubular damage.

Decrease in Glomerular filtration rate (GFR) is responsible for kidney tissue injury this and may be due to presence of stone in urinary tract, which obstruct urine flow and waste products, thereby increase in nitrogenous substances. Treatment with *S. grandiflora* extract showed significant decreased in nitrogenous substances. After treatment with test drug, there is an improvement in GFR due to reduction in kidney tissue injury and inflammation. Higher dose of *S. grandiflora* (400mg/kg) showed reduction in elevated urinary nitrogenous substances. In urolithiasis, ethylene glycol and ammonium chloride administration induces nephrotoxicity which were characterized by marked elevation of serum uric acid, serum urea, serum creatinine and blood urea nitrogen (BUN). Treatment with *S. grandiflora* reduced the accumulation of nitrogenous substances in blood, decreases nephrotoxicity and thereby improved GFR. Higher dose of *S. grandiflora* extract decreases the amount of nitrogenous waste products.

Microscopic examination of kidney sections shows cellular derangement, hypercellularity, necrosis, congested blood vessels and injured glomerulus in lithiatic animals. However, treatment with test extract, notable improvements were observed, particularly of the animals treated with high dose of *S. grandiflora* extract. High level exposure of oxalate and calcium oxalate crystals causes cellular injury because of membrane lipid peroxidation through intracellular reactive oxygen species generation. Therefore, reduction in renal oxidative stress may be an effective approach in the treatment of urolithiasis.

## CONCLUSION

The present investigation supports the use of *Sesbania grandiflora* in traditional medicine against urolithiasis. It is concluded that flavonoid rich fraction of *S. grandiflora* reduced and prevented the growth of urinary stones. It also seems that higher dose of extract shows curative effect. The extract of *S. grandiflora* is helpful to prevent the recurrence of disease. The presence of flavonoid and acids were responsible for antiurolithiatic activity.

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**Source of Support:** The author(s) received no financial support for the research, authorship, and/or publication of this article.

**Conflict of Interest:** The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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