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RESEARCH ARTICLE

# Preclinical Assessment of Antiurolithiatic Activity of *Mangifera indica* Seeds on Ethylene Glycol Induced Urolithiasis Rat Model

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**A B S T R A C T**

*Mangifera indica* seeds (MIS) used traditionally for treating multiple ailments including urolithiasis. In current study aqueous methanolic extract of MIS was examined for antiurolithiatic potential against calcium oxalate (CaOx) crystals. *In vitro* analysis (nucleation, aggregation and growth assays) were performed with 20; 40; 60; 80 and 100 mg/mL concentrations of extract against standard drug (cystone). For *in vivo* analysis CaOx crystals induced by administration of drinking water comprising 0.75% v/v ethylene glycol (EG) and 1% w/v ammonium chloride (AC) for initial 3 days followed by intake of 0.75% v/v EG for next 25 days. Total 36 rats were allocated into 6 groups receiving vehicle, EG + AC, Cystone and extract (250; 500 and 1000 mg/kg) respectively. Urine and blood samples were collected for biochemical analysis. In *in vitro* analysis 1000 mg/mL concentration significantly inhibit crystal formation when compared to standard. While MISE (500 and 1000 mg/kg) produced significant reduction in serum creatinine, BUN and uric acid levels while increased urine volume, Mg, pH and citrate levels of urine in MISE treated rats. This result gives a scientific basis for its traditional claims.



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## INTRODUCTION

Urolithiasis is recognized as the 3rd most common condition of urinary tract which may aggravate some disorders, such as urinary tract infections and prostate complaints (Goyal *et al*., 2017). Some factors that are considered as the root cause of stone formation, such as increased concentration of ions, solute and uric acid in urinary filtrate which makes the urine supersaturated (Tilahun and Beyene, 2018). No current treatment can dissolve the stones and prevent the reoccurrence of stone formation. Generally, it is treated with invasive and interventional methods, while a few drugs such as alkalizers and diuretics are available for treatment. But these methods adhere some severe adverse effects such as hemorrhage, renal fibrosis, infections, costly and require a long follow up (Li *et al*., 2017). The value of plants for mankind has its special place as it provides food, medicine and fulfills various other requirements. The over employment of synthetic agent for treatment results in increased occurrence of adverse effect (Goyal *et al*., 2016). Several studies show the inhibitory potential of several herbs from lithiatic disease such as green tea (*Camellia sinensis*), raspberry (*Rubus idaeus*) and parsley (*Petroselinum sativum Hoffm*) (Gupta and Kanwar, 2018).

*Mangifera indica* seed belongs to family Anacardiaceae; which has traditionally been recommended to reinforce nervous and circulatory system of blood, treatment of anemia, diarrhea, urinary track inflammation, as diuretic, anti-rheumatic, anti-diabetic, anti-asthmatic, anti-syphilis, anti-ulcer and hepatic disorders, astringent, emetic, in toothache and for cough (Sarfaraz *et al*., 2011). Previous studies revealed the presence of numerous bioactive compounds such as, flavonoids and phenolic acids, carotenoids, vitamin C, carbohydrates, proteins, fats, starch and dietary fiber (Cristian *et al*., 2016; Kalpna *et al.*, 2016). The seed extracts have been stated for numerous pharmacological activities such as antioxidant (Ribeiro *et al*. 2008; Maisuthisakul and Gordon 2009), anti-inflammatory (Garrido *et al*. 2004), antidiabetic (Jain *et al*., 2014), and immune-modulatory activity (Garcia *et al*., 2003). The current investigation was undertaken to evaluate the antiurolithiatic potential of *Mangifera indica* seeds extract (MISE) on calcium oxalate (CaOx) stones and

determination of possible mechanisms of action using both *in vitro* and *in vivo* methods.

## MATERIALS AND METHODS

**Collection of plant material and extraction:** MIS were procured from local market in Faisalabad. Identified and authenticated by a Taxonomist "Dr. Mansoor Hameed" of Department of Botany, University of Agriculture Faisalabad and dried sample was deposited to herbarium via voucher no. 79-1-2019. Seeds were manually removed from endocarp, shade dried and ground to coarse powder its 500g was soaked in aqueous methanol (20:80) solvent (2.5 L) for 72 hours with intermittent stirring. Filtered and concentrated by rotary evaporator at 40ºC.

**Qualitative phytochemical analysis:** MISE was evaluated for presence of different groups by standard methods as previously described by Kaplan *et al*. (2016).

**Physicochemical analysis:** Physicochemical analysis of dried powder (loss on drying, total ash, acid insoluble ash and water soluble ash), was performed according to standard United States Pharmacopoeia-National Formulary (2003) methods.

**Antiurolithiatic activity**

***In vitro* evaluation**

**Nucleation assay:** Solutions of calcium chloride ($CaCl_2$) 5 mmol/L, sodium oxalate ($NaC_2O_4$) 7.5 mmol/L and sodium chloride (NaCl) (0.15 mol/L) at 6.5 pH were formed in Tris buffer (0.05 mol/L) at 37ºC. Crystallization was initiated by mixing different concentrations (20, 40, 60 80, 100 mg/mL) of MISE (0.1 mL) and standard (cystone) with $CaCl_2$ (3 mL) and $NaC_2O_4$ (3mL) (Patel *et al*., 2012). Incubated at 37°C for 30 minutes and optical density (OD) was recorded at 620 nm.

**Aggregation assay:** Solution of $CaCl_2$ and $NaC_2O_4$ each 50 mmol/L were placed in water bath for 1 hour at 60ºC. Cooled and incubated for 12 hours to yield CaOx crystals. 1 mg/kg of CaOx crystals was mixed with Tris buffer (0.05 mmol/L), NaCl (0.15 mM) 500 uL each having pH 6.5 and different concentrations of standard and MISE 20; 40; 60; 80; 100 mg/mL (Saha and Verma, 2013). After 30 minutes of incubation OD was recorded at 620 nm.

**Growth inhibition assay:** Solution of $CaCl_2$ (4mM), $NaC_2O_4$ (4 mM), $NaCl_2$ (10 mM) and Tris buffer (10 mM) were mixed having pH 7.2 with CaOx crystals (30uL), different concentrations (20; 40; 60; 80; 100 mg/mL) of MISE and standard, incubation was done at 37°C for 30 minutes. Absorbance was recorded at 214 nm (Chaudhary and Singla, 2008).

***In-Vivo* evaluation**

**Animals:** Albino Wistar rats (175-255 g) were breed in animal house of Government College University, Faisalabad (GCUF) and kept under controlled environment in propylene cages, at a temperature (23±2ºC), 12-hour light/dark cycle, humidity 35-65%, fed at normal pellet diet and water *ad libitum*.

**Ethical approval:** Approval for study design was taken from Institutional Review Board of GCUF bearing reference no. GCUF/IRB/2082 (05-05-2018).

**Experimental design:** Ethylene glycol (EG) 0.75% v/v and 1% w/v ammonium chloride (AC) were given for 3 days to hasten processes of lithiasis, followed by administration of 0.75% EG for next 25 days in drinking water to all rats except normal control. Animals were divided into 6 groups (n=6) and dosed orally.

Group I: Normal control, receiving vehicle only.
Group II: Disease control.
Group III: Cystone (750 mg/kg) was administered.
Groups IV-VI: Treatment groups receiving MISE at 250; 500 and 1000 mg/kg concentrations respectively (Nagar *et al*., 2015).

On 29th day, urine was collected by keeping animal individually in metabolic cage for evaluation of urine volume, calcium, magnesium, citrate and creatinine levels. On 30th day, blood was withdrawn by cardiac puncture. Serum was separated by centrifugation for estimation of uric acid, creatinine, and blood urea nitrogen using randox kits. After weighing animals were killed under ether anesthesia by cervical dislocation, kidneys were removed, weighed and preserved in 10% formalin solution for histopathology. Ice cold saline phosphate buffer used for preparation of kidney tissue homogenate for evaluation of superoxide dismutase (SOD) and malondialdehyde (MDA) levels.

**Statistical analysis:** Results were expressed as mean ± SEM. Two-way analysis of variance (ANOVA) followed by "Bonferroni posttests" was applied using Graph Pad Prism version 5. $P<0.05$ was set as statistically significant.

## RESULTS

**Qualitative phytochemical analysis:** The percentage yield of extract was 35%. Qualitative phytochemical analysis of MISE showed presence of alkaloids and flavonoids, tannins and teiterpene whereas steroids, saponins and phalobatannins were absent (Table 1).

**Physicochemical analysis:** The result showed that MIS contained total ash 2.46%, water soluble ash 8.59%, water insoluble ash 8.12% and 9.2% loss on drying (Table 2).

**Table 1:** Qualitative phytochemical evaluation of MISE

| Phytochemicals identified | Name of chemical test | Inference |
|---|---|---|
| Alkaloids | Dragendroff's test | + |
| Flavonoids | Alkaline reagent | + |
| Tannins | $FeCl_3$ test | +++ |
| Phlobatannins | HCl test | - |
| Triterpene | $H_2SO_4$ test | +++ |
| Steroids | Liebbermann-burchard test | - |
| Saponins | Frothing test | - |

**-:** Absent; +: Trace; ++: Moderately present; +++: excellent

**Table 2:** Physicochemical analysis

| Parameters | Values (%) |
|---|---|
| Total ash | 2.46 |
| Water soluble ash | 8.59 |
| Water insoluble ash | 8.12 |
| Loss on drying | 9.2 |

**Anti-urolithiatic activity**

***In vitro* evaluation:** Kidney stones are mostly comprised of CaOx (70-80%) and calcium phosphate (20-30%). With the passage of time the crystals unite to make a small hard mass that termed as stone. Further, there are two types of CaOx stones that are calcium oxalate monohydrate (COM), and calcium oxalate dehydrate (COD) stones (Saha and Verma, 2013). Whereas the results of current study showed that MISE had better capability to inhibit aggregation of CaOx crystals. The primary step in formation of stones in urinary tract are initiation of crystallization step started after 5 minutes of incubation period and continued for the rest of reaction time. Then the crystals were evaluated for precipitation. In control group there was a gradual rise in size and number of crystals whereas in extract supplemented reaction no increase was noted in crystal size when compared to standard. All the effects were directly proportional to the amount of dose and a significant inhibition of precipitates was observed at dose of 1000 mg/mL when compared to Cystone (Table 3). Concluding, MISE has preventive property to inhibit the nucleation and growth step in crystals formation.

***In vivo* evaluation:** A significant increase in body weight, urine volume, Ca, uric acid and pH was noted in disease control group when compared to negative control. Extract at 1000 mg/kg presented a significant (P<0.001) increase in pH, decreased urinary calcium and uric acid levels while produced an increases in magnesium secretion and creatinine clearance in urine (Table 4).

In disease control, the values of serum creatinine, uric acid and BUN levels were raised significantly (P<0.001) as compared to negative control values. While MISE produces a dose dependent decrease in values of serum creatinine, uric acid and BUN levels (Table 5).

**Histopathological analysis:** In histopathological examination, disease control showed a prominent tubular congestion, necrotic debris, calculi, tubular cell swelling, peritubular inflammation, interstitial edema, and peritubular fibrosis. In standard group, calculi were not present while peritubular fibrosis was reduced. Glomerular atrophy and interstitial edema was not seen. Extract treated groups (250, 500 and 1000 mg/kg) showed reduced number of calculi, less tubular swelling and necrotic debris (Fig. 1).

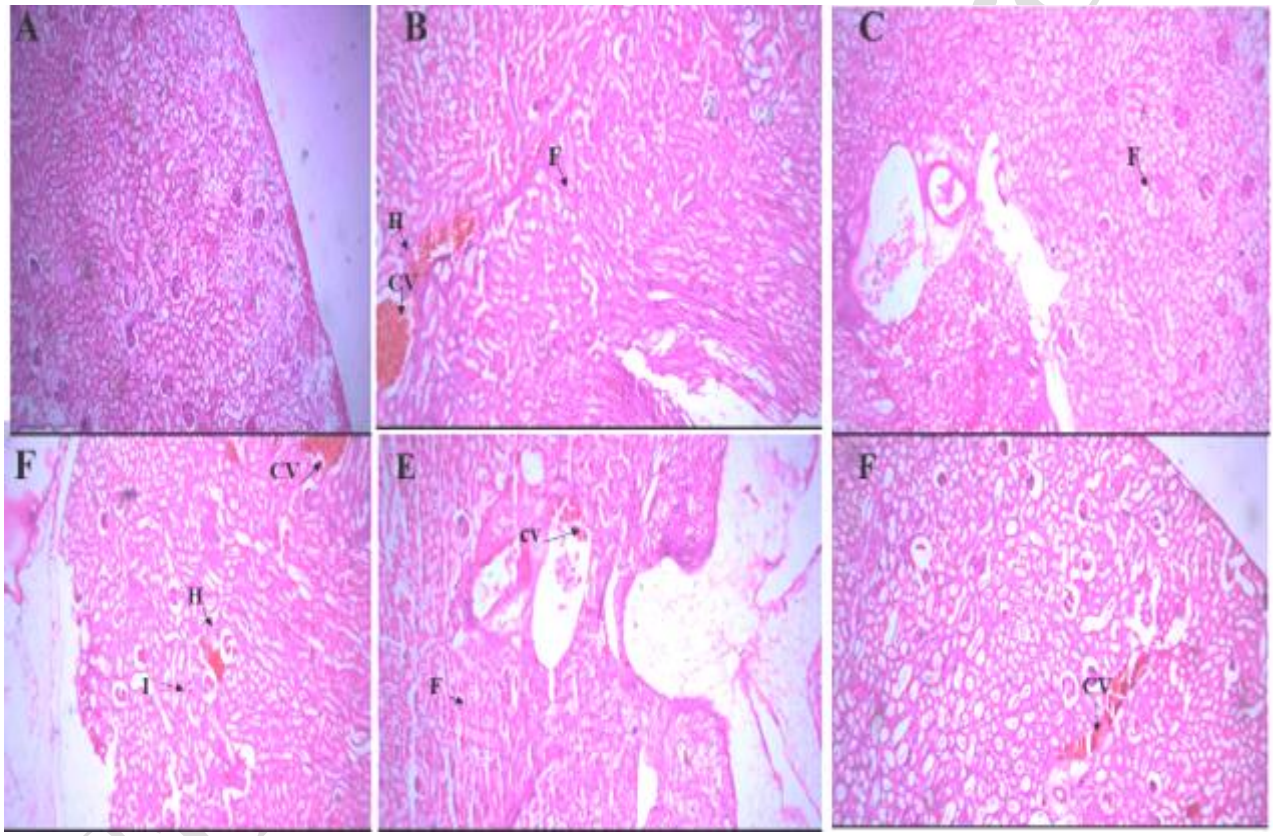


**Fig. 1:** Histopathology (hematoxylin and eosin staining) of kidney sections (40X). (A) Normal control receive vehicle, (B) disease control (hyperoxaluric) receive EG + AC, (C) Standard group (hyperoxaluric): receive Cystone (750 mg/kg). (D-E) Test groups: (hyperoxaluric) rats recive 250; 500 and 1000 mg/kg MISE respectively. CV – congestive vessels; H- hemorrhage; F – fibrosis; I- inflammation.

**Table 3:** Effect of *Mangifera indica* seeds on inhibition of nucleation, aggregation and growth of calcium oxalate crystals

| Concentration | Nucleation assay (%) | | Aggregation assay (%) | | Growth assay (%) | |
|---|---|---|---|---|---|---|
| (mg/mL) | MISE | STD | MISE | STD | MISE | STD |
| 20 | 65±1.73 $^{ns}$ | 72 ±1.16 | 69±1.15 $^{ns}$ | 60±1.15 | 62±1.16 $^{ns}$ | 70±1.16 |
| 40 | 65±1.15* | 74±1.15 | 76±0.88* | 65±1.52 | 70±1.46 $^{ns}$ | 80±2.66 |
| 60 | 88±2.08* | 79±2.72 | 83±2.40** | 70 ±1.15 | 76±1.16 $^{ns}$ | 86±1.99 |
| 80 | 155±2.88*** | 82±1.15 | 102±4.66*** | 76±1.15 | 80±1.10* | 95±0.86 |
| 100 | 194±2.60*** | 85±1.15 | 105±3.20*** | 82±1.15 | 86±1.29* | 101±2.99 |

Values are presented as mean±S.E.M (n=3) STD: Standard, MISE: Mangifera indica seeds extract; ***P<0.001, **P<0.01, *P<0.05 and ns=non-significant (P>0.05) when compared with standard.

**Table 4:** Effect of aqueous methanolic extract of *Mangifera indica* seeds on body and kidney weights and various urinary parameters of urolithiasis

| Parameters | Negative control | Disease control | Standard (750 mg/mL) | MISE (250 mg/mL) | MISE (500 mg/mL) | MISE (1000 mg/mL) |
|---|---|---|---|---|---|---|
| Body weight (g) | 145±2.88 | 115±2.88 | 135±2.88*** | 125±2.88* | 132±1.45*** | 150±5.77*** |
| Kidney weight (g) | 0.41±0.01 | 0.69±0.01 | 0.57±0.01* | 0.64±0.01$^{NS}$ | 0.57±0.00* | 0.54±0.01** |
| Urine volume (mL) | 8.00±0.11 | 17.3±1.45 | 26.0±2.08* | 23.3±0.88$^{ns}$ | 27.3±1.45* | 30.6±2.96** |
| Ca (mg/dL) | 2.43±0.17 | 4.93±0.14 | 2.03±0.17*** | 4.26±0.14*** | 3.80±0.11*** | 2.800±0.11*** |
| Mg (mg/dL) | 3.10±0.11 | 2.20±0.11 | 3.00±0.11*** | 2.40±0.11$^{ns}$ | 2.60±0.11** | 2.700±0.11** |
| Citrate (mmol/L) | 0.92±0.04 | 0.37±0.01 | 1.05±0.12 *** | 0.62±0.01 $^{ns}$ | 1.05±0.07*** | 1.60±0.20*** |
| Creatinine (mg/dL) | 2.34±0.11 | 5.1±0.14 | 1.90±0.11*** | 4.90±0.11 | 3.50±0.11** | 2.01±0.11*** |
| Uric acid (mg/dL) | 5.10±1.11 | 8.90±1.11 | 5.20±0.11*** | 6.90±0.11* | 6.20±0.11** | 5.60±0.11*** |
| pH | 6.23±0.29 | 4.00±0.14 | 7.16±0.14** | 6.36±0.14* | 6.96±0.08** | 7.56±0.33*** |

All values are presented as mean±SEM (n=6); ***P<0.001, **P<0.01, *P<0.05 and ns= non-significant (P>0.05) when compared with disease control.

**Table 5:** Effect of aqueous methanolic extract of *Mangifera indica* on various serum and kidney homogenate parameters of urolithiasis

| Parameters | Negative control | Disease control | Standard (750 mg/mL) | MISE (250 (mg/mL) | MISE (500 mg/mL) | MISE (1000 mg/mL) |
|---|---|---|---|---|---|---|
| Parameters measured in serum | | | | | | |
| BUN (mg/dL) | 19.6±1.45 | 36.0±2.08 | 22.3±1.76 *** | 29.3±2.02** | 22.6±1.76*** | 18.0±1.73*** |
| Serum creatinine (mg/dL) | 0.65±0.07 | 1.13±0.17 | 0.58±0.01*** | 0.93±0.14$^{ns}$ | 0.57±0.02*** | 0.48±0.02*** |
| Creatinine clearance (mL/min) | 0.98±0.11 | 0.49±0.02 | 1.13±0.14*** | 0.91±0.02*** | 0.94±0.01*** | 1.18±0.13*** |
| Uric acid (mg/dL) | 3.46±0.12 | 4.86±0.14 | 3.63±0.12*** | 4.56±0.26$^{ns}$ | 3.40±0.20*** | 3.93±0.18*** |
| Parameters measured in kidney homogenate | | | | | | |
| MDA (nmoL/mg protein) | 0.58±0.02 | 1.30±0.01*** | 0.63±0.02*** | 0.64±0.20*** | 0.61±0.02*** | 0.58±0.00*** |
| SOD (U/mg protein) | 7.00±0.57 | 5.06±0.12*** | 7.83±0.17*** | 7.23±0.12*** | 7.83±0.12*** | 8.70±0.11*** |

All values are presented as mean±SEM (n=6) ***P<0.001 and ns= non-significant (P>0.05) when compared with disease control.

## DISCUSSION

The treatment for urolithiasis still contains many gaps to properly elaborate the establishment of stone formation mechanism, management of diet plan, medicinal agent and herbal treatment (Baskar *et al*., 1996). CaOx was encountered as most common type of stone in urolithiasis. Nucleation, aggregation and growth of crystals were the series of events required for stone formation. The present study was performed to identify the possible mechanism of action for antiurolithiatic effect of MISE against CaOx stones. Nucleation is preliminary step, supersaturated state of filtrate results in precipitation of crystals. Inhibiting of nucleation step was observed with MISE treatment by decaying CaOx crystals into small particles. Presence of extract decreases the continuous increase in size and number of crystals in a concentration-dependent manner when compared to disease control. In process of aggregation, crystals of CaOx begin to aggregate with other crystals, increase in size and cause the renal injury (Manish *et al*., 2011).

The results of present study are consistent with earlier work of Bashir and Gilani (2009) with extract of *Bergenia ligulata* rhizome that caused reduction in growth and aggregation of CaOx crystals (Bashir and Gilani, 2009).

For *in vivo* evaluation of antiurolithiatic activity EG is a commonly used chemical agent. Which metabolizes to glyoxylate, glycolate and oxalate, each of them play a significant role in development of calcium oxalate monohydrate in urine and renal tubules (Bilbault and Haymann, 2016). As crystalluria is pH dependent and nature of stone is predictable by pH of fasting urine (Sujatha *et al*., 2010). Given treatment restored normal pH of urine. Dissolution of calcium and oxalate complexes was may be due to rise in pH.

Plants with antilithiatic property may cause diuresis for stone dissolution (Gupta and Kanwar, 2018). Activity of MISE may be hidden in its diuretic potential is ascribed due to presence of potassium and tannins (Cristian *et al*., 2016). Similarly, presence of citrates decreased the super saturation of CaOx and phosphate stone through formation of soluble citrate complexes. Metabolic abnormality of hypocitraturia is mostly seen in lithiatic patient (Gupta and Kanwar, 2018). In current study treatment of EG decreased levels of urinary citrate while a dose dependent increase was seen in all MISE treated groups.

Several stone promoting (calcium and uric acid) and inhibiting (magnesium) dynamics influence the stone formation (Basavaraj *et al*., 2007). Current study revealed elevation of calcium and significant decrease in magnesium levels in urine in EG treated rats when compared with normal control as presented in Table 4. Treatment with 500 and 1000 mg/kg of MISE produced significant decrease in urinary calcium and improved the urinary creatinine clearance. The highest reduction in uric acid was recorded at 1000 mg/kg in serum as well as in urine that eventually altered crystal growth formation.

Urolithiasis produces impairment of tubular and renal function that can be observed with elevation of serum creatinine and BUN (Orlic *et al*., 2014). All treatment groups except 250 mg/kg produced significant upsurge in creatinine clearance, while decrease serum creatinine and BUN values.

Generation of ROS results in epithelial injury that proliferate the area for crystal attachment and assists the steps such as nucleation and aggregation through saturating urine in renal tubules (Selvam and Bijikurien, 1992). Previous studies suggested that administration of vitamin E prevent the precipitation of crystals in kidney tissues, decreasing the urinary elimination of calcium and oxalate in urolithiatic patients (Huang *et al*., 2002). SOD levels increased and MDA levels decreased dose dependently in all the treatment groups as compared to that of disease control. MISE at 1000 mg/kg had an optimal antioxidant activity and restored the normal levels of SOD and MDA levels. Polyphenols and vitamin E present in extract may be responsible for this effect.

In histopathological examination the disease control group showed accumulation of calcium oxalates, interstitial hemorrhage, congested blood vessels and

interstitial fibrosis. Treated rats showed significant reduction in the number and size of CaOx deposition in different areas of renal tubules along with reduction in extent of damage caused by the ethylene glycol to kidney tissues. The preservation effect was more significant at concentration of 1000 mg/kg of extract. This effect may be observed due to the occurrence of antioxidant metabolites that give protective effect to the kidney from oxidants.

**Conclusions:** Our finding indicated that MISE had potential for inhibition of CaOx crystal formation and promoting its dissolution. Similarly, the extract prevented the formation of CaOx crytals in rat's kidney possibly due to restoring the normal antioxidant levels, diuresis, hypocalciuria, hypermagnesemia, urinary alkalanization and hypercitraturia.

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**Authors contribution:** SI and UZ designed and execute the experimental work and analyzed the obtained data. BA help in data collection.

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