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Studying inhibition of calcium oxalate stone formation: an *in vitro* approach for screening hydrogen sulfide and its metabolites

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ABSTRACT

Purpose: Calcium oxalate urolithiasis is one of the most common urinary tract diseases and is of high prevalence. The present study proposes to evaluate the antilithiatic property of hydrogen sulfide and its metabolites like thiosulfate & sulfate in an in vitro model.
Materials and Methods: The antilithiatic activity of sodium hydrogen sulfide (NaSH), sodium thiosulfate ($Na_2S_2O_3$) and sodium sulfate (Na_2SO_4) on the kinetics of calcium oxalate crystal formation was investigated both in physiological buffer and in urine from normal and recurrent stone forming volunteers. The stones were characterized by optical and spectroscopic techniques.
Results: The stones were characterized to be monoclinic, prismatic and bipyramidal habit which is of calcium monohydrate and dihydrate nature. The FTIR displayed fingerprint corresponding to calcium oxalate in the control while in NaSH treated, S=O vibrations were visible in the spectrum. The order of percentage inhibition was $NaSH>Na_2S_2O_3>Na_2SO_4$.
Conclusion: Our study indicates that sodium hydrogen sulfide and its metabolite thiosulfate are inhibitors of calcium oxalate stone agglomeration which makes them unstable both in physiological buffer and in urine. This effect is attributed to pH changes and complexing of calcium by $S_2O_3^{2-}$-and SO_4^{2-}- moiety produced by the test compounds.

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INTRODUCTION

The incidence of urolithiasis in recent times is alarmingly increasing in both adult and pediatric populations (3 per 1000 in men and 2 per 1000 in women) (1, 2). This may be due to the change in lifestyle and dietary intake as diet plays an important role in the pathogenesis of kidney stone (3). Recurrent stone formation is one of the major concerns in this disease where frequent medical cares for the patients are required (4). Even though calcium phosphate and Mg-ammonium phosphate stones are prevalent, calcium oxalate stones are occurring with high incidence (70-80%) (5). This may be due to the relatively high consumption of animal protein and fat and low consumption of carbohydrate in the diet (1).

The formation of stones nadir due to calcium oxalate crystal retention in the kidney resulted from the accumulation plasma oxalate, derived from both endogenous and exogenous sources. Experimental evidence indicates that tolerance of kidney stone formation varies among the animals (6). Adhesion of newly formed Calcium oxalate monohydrate (COM) crystals to the apical surface of renal tubular epithelial cells could be an important initiating event of stone formation (7). Interaction of renal epithelial cells with COM crystals has been shown to increase the generation of reactive oxygen species and are responsible for damage renal tubules (8). However, in animal experimental models, it is difficult to discriminate between effects caused by crystals or by oxalate as calcium oxalate crystalluria cannot exist without hyperoxaluria. Hence in this study we used one *in vitro* experimental model to study the effect of the drug.

Dietary management and medical expulsion therapy such as lithotripsy, ureteroscopy, shock wave lithotripsy (SWL) and percutaneous nephrolithotomy (PNL) are some of the medical management procedures for renal stones. However, most of these approaches have significant side effects and this leads to the stimulation for alternative therapy in this field.

All these facts indicate the need for new therapeutic target or agent for the treatment of renal stones (3. 4). Recent studies have proved that anti-oxidants, thiazide diuretic, thiol based agents are few promising agents that can be used to reduce Calcium oxalate crystal induced renal injuries (9-11). They primarily reduce urinary calcium excretion and thereby inhibit the formation of calcium containing stones.

Sodium thiosulfate, promising anti-urolithiatic agent received considerable attention as a drug and its clinical trial on recurrent stone formers is an evidence for sulfur based drugs for the treatment of renal stone. Antioxidant potential and its ability for sulfur group donation underline the effectiveness of thiosulfate in renal stone treatment (9, 10). The metabolites of thiosulfate, namely, hydrogen sulfide and sulfate are also reported to have similar property, but without scientific evidence as anti-urolithiatic agent (12, 13). In this manuscript, we compare the effectiveness of thiosulfate, hydrogen sulfide and sulfate in inhibiting *in vitro* crystallization process in physiological buffer, normal and pathological urine.

MATERIALS AND METHODS

Chemicals

The chemicals used in this study were purchased from Hi media®, India except Sodium hydrogen sulfide, bought from Sigma-Aldrich®.

In Vitro calcium oxalate synthesis

In vitro calcium oxalate was synthesized according to the procedure described by Hennequin et al. with some minor modifications (14). Calcium oxalate was prepared by measuring equal volume of stock solutions of 5 mM calcium chloride ($CaCl_2$) and 0.5 mM sodium oxalate ($Na_2C_2O_4$) prepared in buffer containing 10 mM Tris-HCl and 90 mM NaCl at pH 6.5 and maintained at 37°C. The resulting white turbid solution was stirred at 400 rpm for 24h and left without shaking for the crystals to settle down. The supernatant was discarded and the crystals were washed twice with ethanol followed by water and subjected to lyophilization. The inhibitory effect of H_2S and its metabolites were analyzed by adopting similar procedures in the presence of trisodium citrate ($Na_3C_6H_5O_7$), sodium hydrogen sulfide (NaSH), sodium thiosulfate ($Na_2S_2O_3$) and sodium sulfate (Na_2SO_4) at equimolar concentrations.

Characterization of crystals by FTIR

The dry crystal morphology was characterized in the absence and presence of test compounds by microscopy using inverted phase contrast microscope (Carl-Zeiss AXIO®) for crystal habit identification at 40X magnification and confirmed with Fourier Transform Infrared spectroscopy using PerkinElmer® (15, 16).

Urine sample collection

All the procedures involving human subjects were approved by the Institutional Ethical committee (IEC) of SASTRA University. A total of 8 volunteers (5 men and 3 women) with a mean age of 42, with a calcium stone forming tendency but having a normal renal function formed the experimental group and 6 volunteers (3 men and 3 women) with a mean age of 38, without any medical co-morbidities or history of urolithiasis formed the control group. The required multiple urine collections were made with their willingness and consent.

Kinetics of calcium oxalate formation in buffer system and urine

The influence of hydrogen sulfide (H_2S) & its metabolites on the kinetics of calcium oxalate formation was studied both in the buffer system as well as in the urine obtained from normal volunteers and recurrent stone formers as per the method explained by Hennequin et al. (14) with some minor modifications in a 48 well plate.

For kinetic study in buffer, solutions of $CaCl_2$ and $Na_2C_2O_4$ were prepared at the final concentration of 3.5 mM and 0.5 mM, respectively in Tris-HCl buffer (0.02 M) containing NaCl (0.15 M) adjusted to pH 6.5. The solutions were mixed in the absence and presence of sodium hydrogen sulfide (NaSH), sodium thiosulfate ($Na_2S_2O_3$) and sodium sulfate (Na_2SO_4) at concentrations ranging from 0.44 mM to 3.5 mM. Trisodium citrate ($Na_3C_6H_5O_7$) was used as the positive control. Crystallization was initiated by adding 100 μL of $Na_2C_2O_4$ in 100μL of $CaCl_2$. All the reactions were carried out in triplicate maintaining the temperature at 37°C and monitored at 620 nm every 1 min using Biotek Micro plate spectrophotometer associated with Gen5™ data analysis software. The percentage inhibition by the test compounds was calculated as per the expression $(1-(T_{si}/T_{sc}))$ X 100, where T_{sc} was the turbidity slope of control and T_{si} the turbidity slope in the presence of the test compounds.

A similar procedure was adopted for urine sample except pH was adjusted to 6.5 and the treatments were added directly to urine excluding $CaCl_2$. The crystallization was initiated by adding 100 μL of $Na_2C_2O_4$ to 100μL of urine. The rest of the procedures were the same as adopted for the buffer system.

Statistical analysis

Data were expressed as mean±S.D. of three observations and analyzed by two way-ANOVA with 95% confidence interval limits to estimate the differences between the test compounds and concentrations using Graph Pad Prism version 5.01.

RESULTS

Crystal morphology: Figure-1 displays the crystal nature in the buffer system as well as in the urine of both normal volunteers of recurrent stone formers. Crystals predominantly resembled the agglomerated form of calcium oxalate dendrites and monoclinic prisms in the buffer system. The agglomeration was found to be reduced in the presence of H_2S & its metabolites. In the urine of recurrent stone formers, bipyramidal weddellite type of stones were observed. Unlike the observations above, H_2S & its metabolites did not change the morphology of the crystals but crystal number was reduced.

FTIR spectroscopy: Figure-2 displays the typical FTIR spectra of synthesized calcium oxalate alone

Figure 1 - Calcium oxalate crystal morphologies representing the type of crystal in a buffer and in b urine at 40 X magnification.

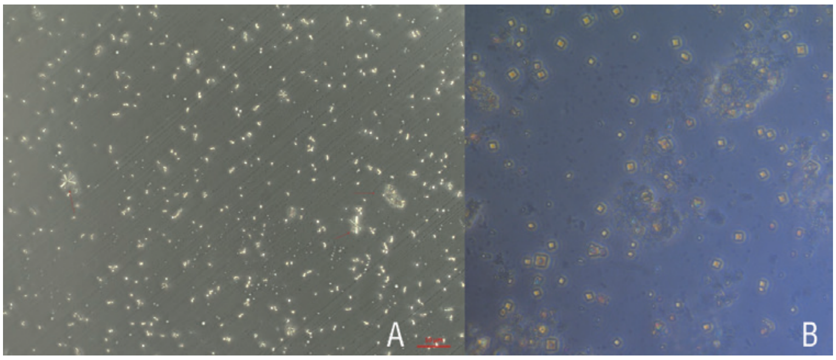
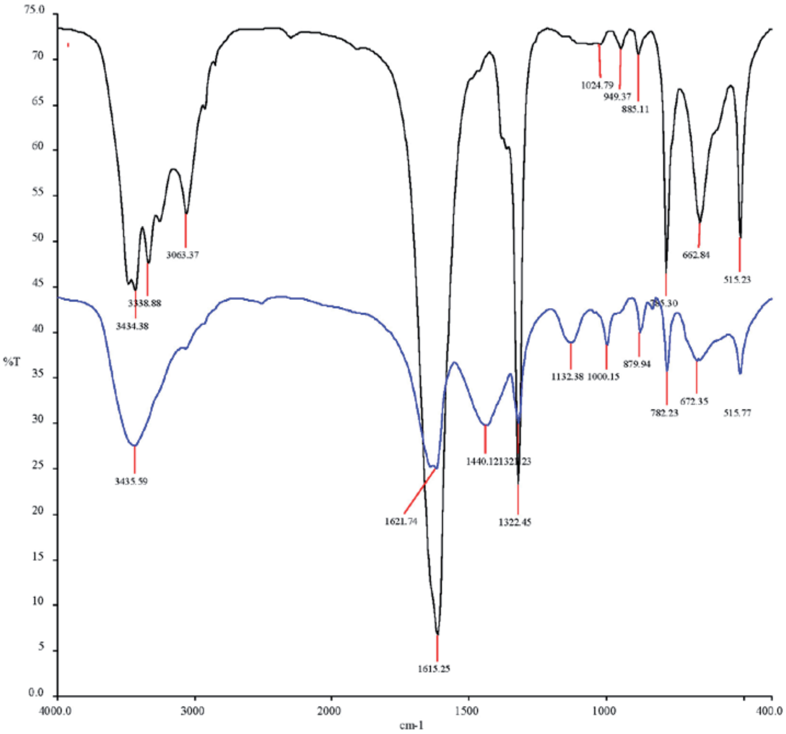


Figure 2 - FTIR spectral data of calcium oxalate obtained in the absence (above) and presence (below) of NaSH.



and in the presence of test compound NaSH. The IR spectra were recorded in the range of 400-4000 cm^{-1} .The absorption bands identified for calcium oxalate were at 3434.26 cm^{-1}, 3063.40 cm^{-1} (Symmetric and asymmetric O-H stretching), 1615.18 cm^{-1}, 1322.44 cm^{-1} (C=O, C-O stretch), 949.36, 885.12 cm^{-1} (C-C stretch), 785.30 cm^{-1}, 662.89 cm^{-1} (out of plane O-H bending and C-H bending) and 515.23 cm^{-1} (O-C--O in plane bending). In the presence of NaSH, the stones obtained had an additional absorption bands at 672.35 cm^{-1} 1000.14 cm^{1}, 1132.38 cm^{-1}, 1440.12 cm^{-1} and 1621.74 cm^{-1}, indicating the presence of SO_4^{2-} and $S_2O_3^{2}$- functional groups, thereby confirming the interaction of H_2S on crystal formation.

Kinetics of calcium oxalate formation in the buffer system: Figure-3 shows the effect of trisodium citrate, sodium hydrogen sulfide, sodium thiosulfate and sodium sulfate on the growth kinetics of calcium oxalate in the buffer system. On comparison with control (absence of test compounds), the percentage inhibition shown by test compounds at 0.44 mM was 38% for NaSH, 9% for $Na_2S_2O_3$ and no significant inhibition with Na_2SO_4 and $Na_3C_6H_5O_7$. At 0.88 mM, the percentage inhibition was 52% for NaSH followed by 31%, 23% and 3% for $Na_3C_6H_5O_7$, $Na_2S_2O_3$ and Na_2SO_4 respectively. For higher concentrations of test compounds at 1.75 mM and 3.5 mM, the increase in percentage inhibition ranged from 48-72% for $Na_3C_6H_5O_7$, followed by 54-68% for NaSH and with almost constant inhibition for $Na_2S_2O_3$ (34-38%) and Na_2SO_4 (10-13%).

Kinetics of calcium oxalate formation in the urine: Figure-4 and Figure-5 display the effect of trisodium citrate, sodium hydrogen sulfide, sodium thiosulfate and sodium sulfate on the growth kinetics of calcium oxalate in the urine of normal and recurrent stone formers.

The urine from the normal volunteers with no previous incidence of stone formation was used

Figure 3 - Effect of test compounds on kinetics of calcium oxalate in buffer. Data are mean±S.D. of three observations. (P<0.001).

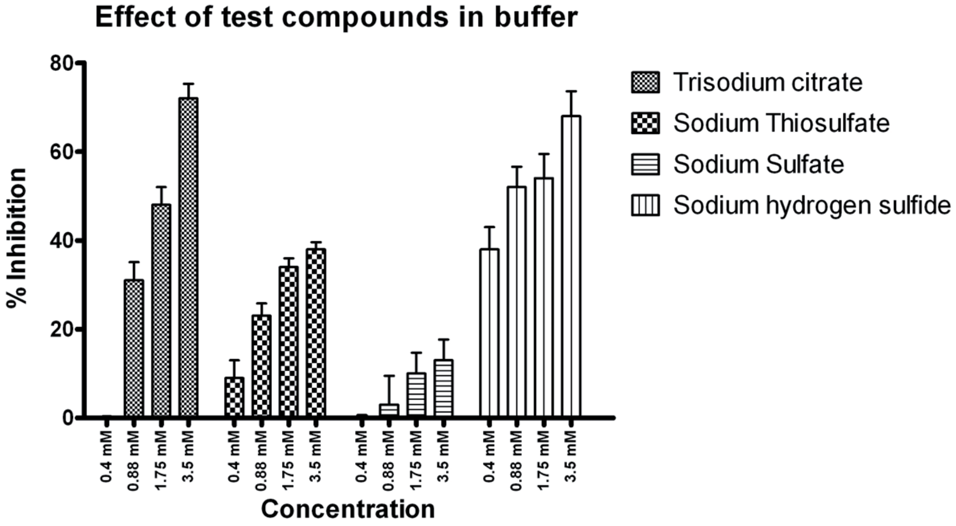
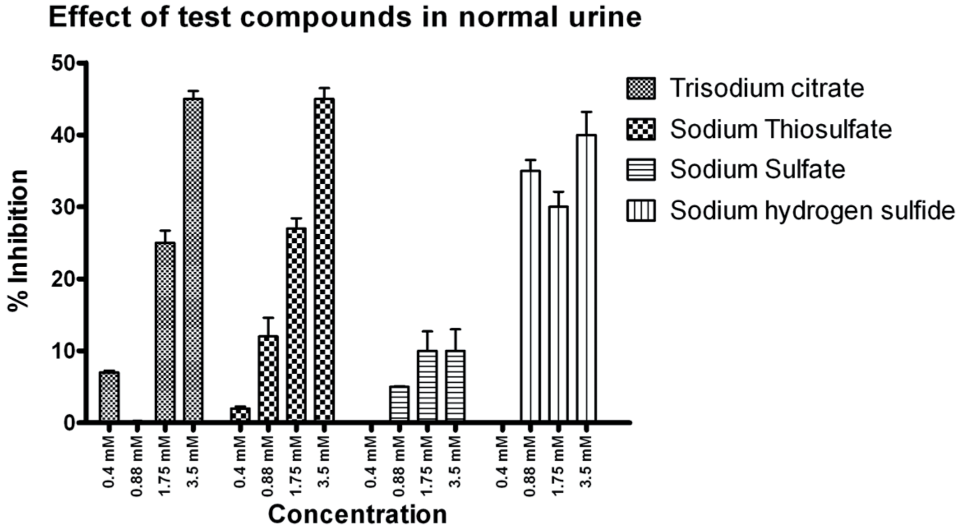


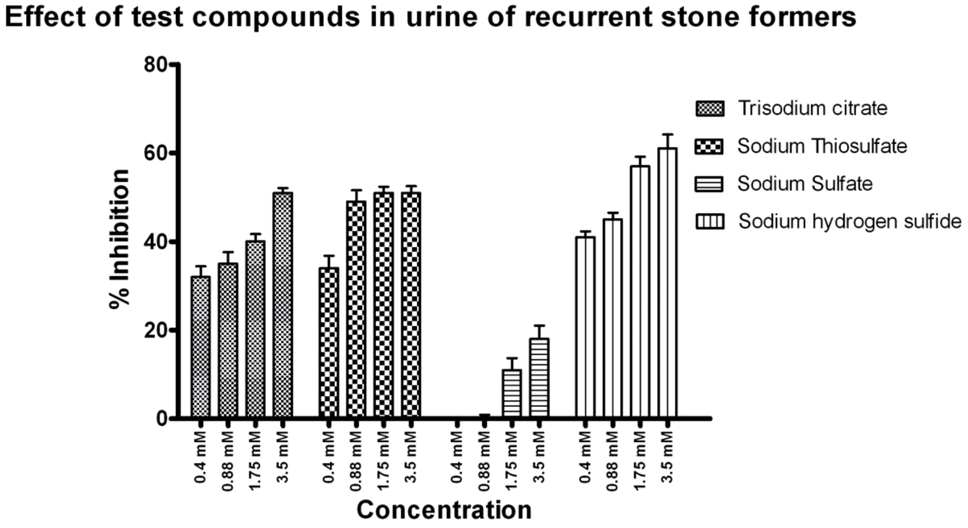
Figure 4 - Effect of test compounds on kinetics of calcium oxalate in normal urine. Data are mean±S.D. of three observations. (P<0.001).



for analysis. The percentage inhibition shown by test compounds at 0.44 mM and 0.8 mM were insignificant compared to control where NaSH showed 35% inhibition. But at a higher concentration of 1.75 mM and 3.5 mM, the percentage inhibition elevated to 25-45% for $Na_3C_6H_5O_7$, followed by 27-45% for $Na_2S_2O_3$ and with almost constant inhibition for NaSH (30-40%) and Na_2SO_4 (10%).

The calcium oxalate crystals studied with urine from stone forming volunteers showed inhibition of 41% for NaSH, 34% for $Na_2S_2O_3$, 32% for $Na_3C_6H_5O_7$ and almost no inhibition at 0.4 mM for

Figure 5 - Effect of test compounds on kinetics of calcium oxalate in urine of recurrent stone formers. Data are mean±S.D. of three observations. (P<0.001).



Na_2SO_4. At higher concentration of test compounds at 0.88 mM, 1.75 mM and 3.5 mM, the percentage inhibition was 45-61% for NaSH, followed by 35-51% for $Na_3C_6H_5O_7$ and with almost constant inhibition for $Na_2S_2O_3$ (49-51%) and Na_2SO_4 (11-18%).

DISCUSSION

The prevalence of urolithiasis has been a major problem affecting people of all socioeconomic levels irrespective of region, age, and gender. Among the four major types of stones, calcium, uric acid, struvite and cysteine, the calcium oxalate accounts for more than 80% of reported cases (17). The mechanism behind the initial growth of crystal still remains an oblivion although many reports suggest that a single nucleus, the "Randall Plaque" is responsible for growth of calcium oxalate crystal (18). The surgical removal of stones by ESWL, ureteroscopy and percutaneous lithotripsy still remains the major treatment strategy despite the fact that recurrence is a major limitation to these procedures (4). Lack of drug treatments targeted towards stone formation, adverse effects of existing drugs such as thiazide diuretics and low efficacy of citrate therapy has kept this area wide open for research.

The current investigation is targeted at testing the influence of sodium hydrogen sulfide, sodium thiosulfate and sodium sulfate, which are products of endogenous metabolism of hydrogen sulfide within the cells, on the formation of calcium oxalate stone (19). The basis of this present study triggers the fact that despite thiosulfate being used clinically, the mechanism of its action remains elusive. Being a part of the endogenous hydrogen sulfide metabolism, if similar activity exists with its metabolites as reported in vascular calcification remains a question to be answered (20). In this study the inhibitory potency of the test compounds was tested on the kinetics of calcium oxalate formation *in vitro* both in buffer and in urine obtained from normal and recurrent stone forming volunteers. In the buffer system, NaSH, the H_2S donor showed 68% inhibition at the highest concentration of 3.5 mM which was 4% less than the positive control $Na_3C_6H_5O_7$. This effect was significant even at the least concentration of 0.44 mM for NaSH (38%) while the positive control showed no inhibition.

On the other hand, chemically proven drug for urolithiasis, thiosulfate showed formation of kidney stone generally under the influence of pH; alkaline pH favors calcium phosphate type of stones while acidic pH favors oxalate, uric acid stones (21).The solution of NaSH was tested to be alkaline (pH=10.9) and immediately increases the pH of the microenvironment and thus might contribute to inhibition of stone formation even at lower concentration as suggested from the kinetic data (Figure-3). However $Na_2S_2O_3$ and Na_2SO_4 in solution were slightly acidic and did not prevent the stone formation in the *in vitro* buffer system.

Evidence from previous report suggest that calcium, sodium, oxalate, urate, Tamm- Horsfall protein and low urine pH are the factors that favors the stone formation and are widely present in stone forming patients (17, 22, 23). The normal calcium oxalate nucleation procedure was not followed in normal urine as its pH was around 7.2 and did not favor the nucleation. Hence urine from normal person was adjusted to pH 6.5 and crystal nucleation was initiated suggesting the pH as a major factor influencing calcium oxalate stone formation as reported by others (21). In recurrent stone formers urine, the maximum inhibition in the nucleation showed by NaSH (61%) which was near consistent with that of buffer system while it was lowered by 20% in normal urine suggesting a low tendency of stone formation in normal subject and the suitability of using a buffer system for the essential evaluation of anti--urolithiasis. On the other hand, $Na_2S_2O_3$ showed 49-51% inhibition in pathological urine similar to that of positive control $Na_3C_6H_5O_7$ indicating its efficacy and agreement with the previous reports (23). Tri sodium citrate and sodium thiosulfate acts by interfering the stone formation through complexing the calcium as suggested by Yatzidis, 1985. Sodium sulfate was found to be a poor inhibitor of stone formation *in vitro*, being the end product of H_2S metabolism that is easily excreted in urine unchanged.

The prepared calcium oxalate crystal morphology was analyzed and assessed according to the guidelines described by Thongboonkerd, 2006. Interestingly, we found two different types of calcium oxalate crystals in buffer & urine. In buffer system the crystals formed was monoclinic and aggregated to form dendritic crystals of calcium oxalate of monohydrate in nature (24). Addition of test compounds reduced the number of agglomerates but not the morphology. However in urine collected from recurrent stone formers, dihydrate stones were predominant as evident from their bipyramidal nature (Figure-1). In general, calcium oxalate stones exist in three forms: monoclinic monohydrate, tetragonal dihydrate and triclinic trihydrate of which monohydrate is thermodynamically stable and forms the majority of kidney stones (24).

FTIR fingerprint spectra of calcium oxalate, showed the characteristic bands of 672.35 cm^{-1}, 1000.14 cm^{-1}, 1132.38 cm-^{1}, 1440.12 cm^{-1} and 1621.74 cm^{-1} suggesting the existence of S=O stretching and bending vibrations pertaining to $S_2O_3^{2-}$ and SO_4^{2-} functional group. This suggests the interference of NaSH on calcium oxalate nucleation, thereby preventing its growth. Further in vivo studies have to be carried out for confirming the same.

CONCLUSIONS

The current study revealed the anti-urolithiatic activity of H_2S & its metabolites in an *in vitro* model. Moreover the effect was observed both for monohydrate and dihydrate forms preventing their aggregation which promotes thermodynamic stability.

CONFLICT OF INTEREST

None declared

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