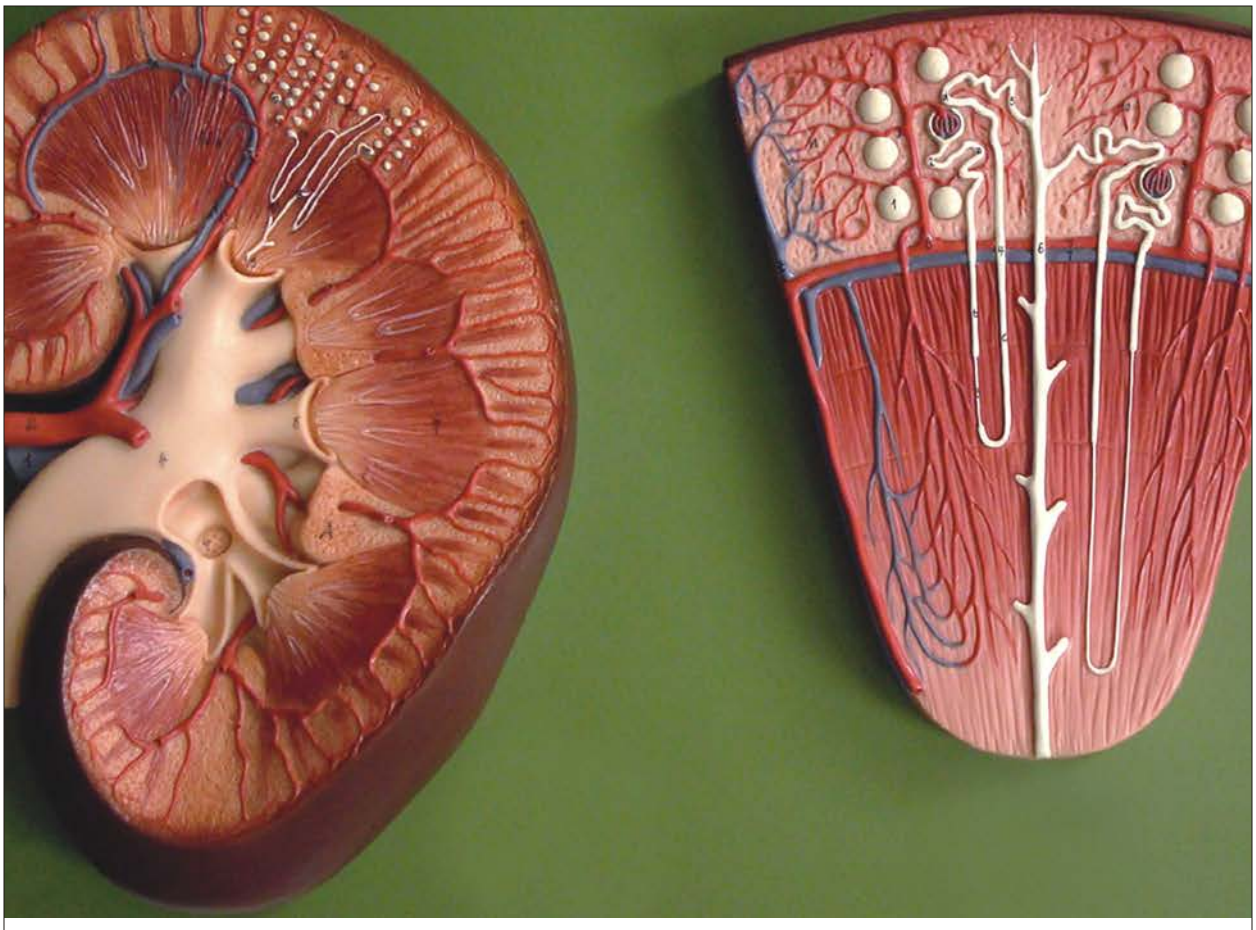

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Research Article
Scanning Electron Microscopy Analysis of Effect of *Pedalium murex* (L.) Seeds on the Morphology of Calcium Oxalate Crystals

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Abstract

Background and Objective: Calcium oxalate crystals are found in majority of kidney stones as calcium oxalate monohydrate (COM) as one of the primary types of kidney stones. The present investigation aimed to better understand the role of *Pedalium murex* Linn. seeds on the inhibition of calcium oxalate (CaO_X) crystals by Scanning Electron Microscopic (SEM) analysis. **Materials and Methods:** Under *in vitro* condition, the effect of *Pedalium murex* Linn. extract on the morphology of CaO_X crystals was studied by scanning electron microscopy. **Results:** Extract inhibited the crystallization of CaO_x, less and smaller particles were observed in the presence of extract. Scanning Electron Microscopic (SEM) images revealed aggregation of crystals without plant extract. With plant extract, the scanning electron micrographs showed discernible crystal unit boundaries. **Conclusion:** *Pedalium murex* Linn. extract was observed to have decreased crystal size and prevented the aggregation of calcium oxalate crystals.

Key words: Calcium oxalate, morphology, *Pedalium murex* Linn., kidney stones, SEM

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Urolithiasis is the process of forming stones in the kidney, bladder and urethra (urinary tract). Kidney stone disease, is a common medical problem all over the world. It is well-known that urinary crystals are the building blocks of stones and urinary super saturation is an initial driving force of promoting the formation of calculi[1]. The stones themselves are also called renal calculi. Globally, the major type of urinary calculi is calcium oxalate (CaO_x)[2,3]. Quantification of the potential for CaO_x to crystallize in the urine, therefore, would provide adequate clinical utility for estimating the risk of kidney stone formation[4]. Scanning Electron Microscopy (SEM) has been used to analyze the compositional and architectural variations of kidney stones retrieved from patient. The process of stone formation starts with precipitation of crystals, when the urine is oversaturated with calcium oxalate. However, urine contains inhibitor(s) that can withhold the process of super saturation. Most of this activity can be ascribed to macromolecules and a considerable participation by citric acid and some ions[5]. After urinary super saturation, multiple steps are involved in the formation of the crystals, which are nucleation, growth and aggregation[6]. The stone formation begins from the formation of nuclei from super saturated urine. The process of crystals in solution attracted each other to form larger particles called as aggregation. Newly aggregated crystals may combine to form a small, hard mass called as stones and the stage is referred to as subsequent growth of crystals[7]. Association of grown crystals with the epithelial cells lining in the renal tubules are called as crystal retention. The retained crystals are combined and form a nidus which finally develops as stones[8,9]. The nucleation step was prevented by urinary macromolecules by adsorption on a crystal surface to induce degradation and dissolution of crystals faces and edges[10]. Only Scanning Electron Microscopy (SEM) can investigate the crystal morphology and its modifications. SEM is the fundamental instrument to investigate micro and nano-structured materials. Based on the previous studies, SEM investigation can give the morphology of crystals at micro and nano-meter scale. In this study, SEM was used to study the crystal morphology of CaO_X crystals[11]. *Pedalium murex* (L.) is a luscious herb, commonly called as 'Yanai Nerunji' in Tamil Nadu, India, is mainly used in the treatment of disorders of urinary systems such as gonorrhea, incontinence of urine, dysuria and removes stone in the bladder[12,13]. In the present investigation, CaO_X crystals were grown in the urine sample and the effect of alcohol extract of *Pedalium murex* (L). seeds was studied on the inhibition of CaO_X crystals.

MATERIALS AND METHODS

All the chemicals, including analytical grade calcium oxalate and solvent were purchased from Sigma Aldrich.

Scanning electron microscope: The CaO_x crystals were analyzed using a VEGA3 TESCAN (Czech Republic) Scanning Electron Microscope (SEM). For the Scanning Electron Microscope (SEM) analysis, samples were coated with a 5 nm-thick gold layer in a sputter coater system. The conductive coating is important because it prevents the charging of the specimen surface during the observation with the SEM, especially when the microscope is operated at high vacuum[14].

Collection of plant material: The *Pedalium murex* (L.) seeds was collected from Thanjavur, Tamil Nadu, India during the month of May-June, 2017 and it was authenticated by Rev Dr. S. John Britto SJ, Director, The Rapinat Herbarium and Centre for Molecular Systematic, St. Joseph College (Autonomous), Tiruchirapalli, Tamil Nadu, India.

Preparation of extracts: The dried seeds of *Pedalium murex* were pulverized and extracted using ethanol in Soxhlet apparatus. Ethanol was used for the process as it is safe for ingestion and also can be evaporated after extraction. The extract was concentrated to dryness at room temperature 34°C to get dried extract. The dried extract was diluted with distilled water to 50 g L^{-1}, which was used for further analysis.

Collection of urine sample: Twenty-four-hour urine specimens were collected from a healthy man, who had no history of kidney stone disease. The samples were refrigerated without preservative for the duration of the collection.

Study without inhibitor (without plant extract): A mixture of 150 mL of urine sample and 50 mL of 0.05 M calcium oxalate was taken in a beaker. After vigorous shaking, the solution was covered with a film and left undisturbed for 48 h. Crystals were centrifuged 3000 rpm for 30 min. The supernatant was discarded and the crystals were transferred to a specimen plate for SEM analysis.

Study with inhibitor (with plant extract): A mixture of 150 mL of urine and 50 mL of 0.05 M calcium oxalate solution was taken in a beaker. After vigorous shaking, the solution was covered with a film and left undisturbed for 48 h. After 48 h, 50 mL of seed extract solution was added in the above

mixture. The whole solution was covered with a film and left undisturbed for 24 h. Crystals were centrifuged 3000 rpm for 30 min. The supernatant was discarded and the crystals were transferred to a specimen plate for SEM analysis.

GC-MS analysis: The JEOL GCMATE II GC-MS with data system is a high resolution, double focusing instrument was used to study the presence of phytochemicals in *P. murex* seeds extract. Ethanol extract was used for the GC-MS analysis.

RESULTS AND DISCUSSION

Crystalline calcium oxalate monohydrate (COM) is the most common material that create kidney stones. The main findings of the present study were that extracts from *P. murex* seeds inhibited the crystallization of CaO_x in solution. Figure 1a showed maximum number of CaO_x crystals and the CaO_x crystals are easily identified by their heterogeneous shape (Fig. 1b-e). Figure 1a showed maximum number and

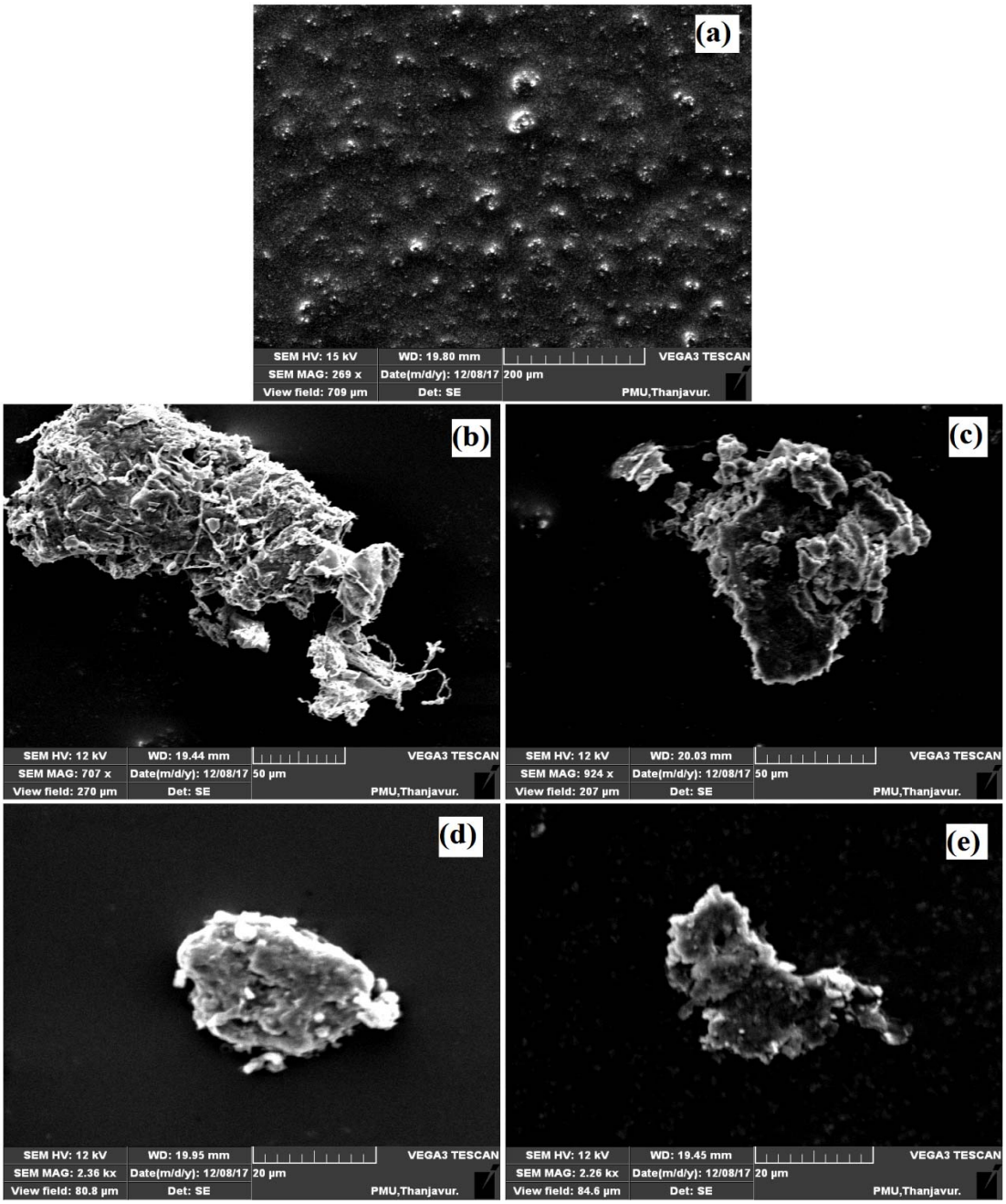

Fig. 1(a-e): SEM images of urine sample along with CaO_x crystals (without plant extract)

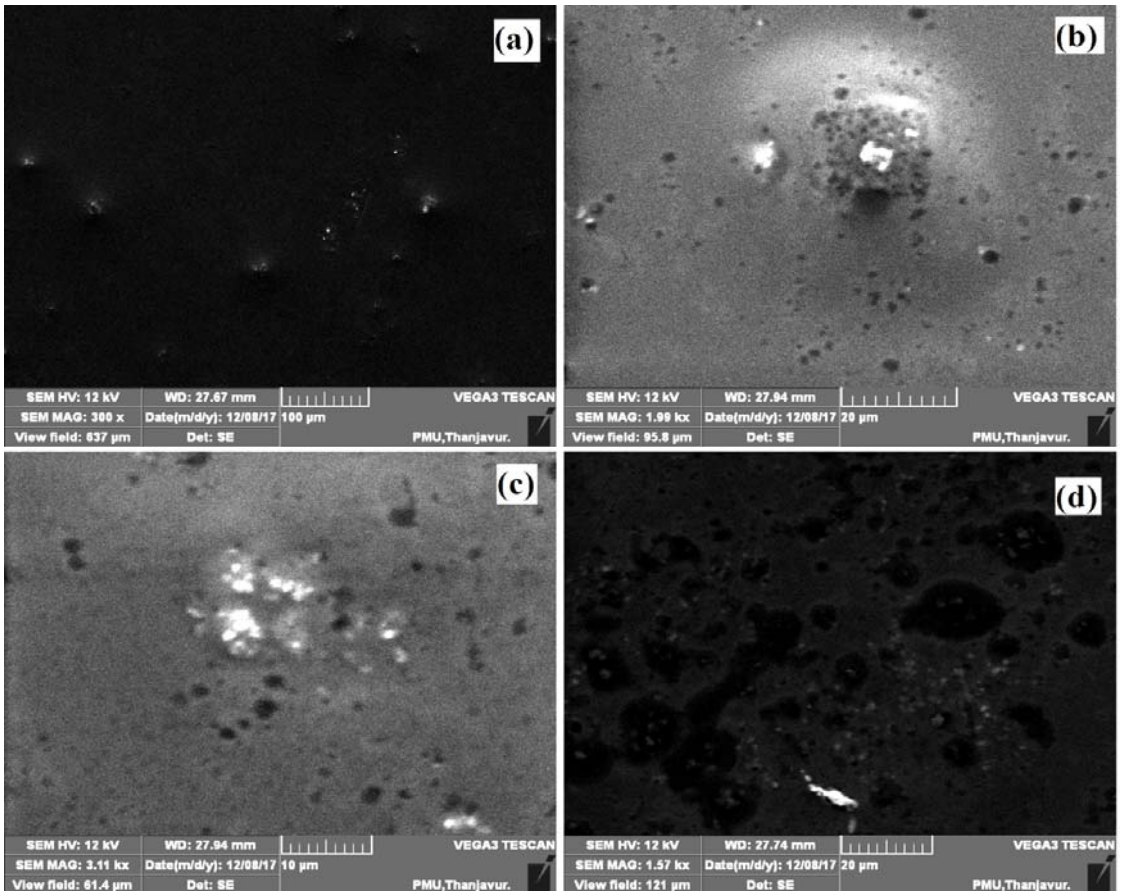

Fig. 2(a-d): SEM images of urine sample with *Pedalim murex* extract. Images clearly showed that, in the presence of plant extract, the length and width of the CaO_x crystals were reduced, dissociated and dissolved

largest size of crystals (without extract), while Fig. 2a showed comparatively less number and smaller size of crystals (with extract). There were less and smaller particles with increasing concentrations of extract as shown in various SEM micrographs (Fig. 2a-d). The seed extract had dissociated (Fig. 2b and c) and dissolved (Fig. 2d) the CaO_x crystal growth. The extract of seed causes fewer numbers of crystals in solution (Fig. 2a), thereby reduced super saturation and the size of the particles. This property of the extract is, therefore, advantageous in preventing urinary stone formation by inducing the excretion of small particles from the kidney and reducing the chance of retention in urinary tract. Further, the seeds of *Pedalium murex* is considered to be diuretic[14-16] and diuretic effects may also reduce stone development when total fluid intake and output increased and such effects have been attributed to several herbal preparations. Very similar results to this study were obtained by Carvalho and Vieira[17]. In present study, the crystals were examined optically and with X-ray diffraction and morphology was found to be closely related to relative super saturation.

Plant extracts may contain substances that inhibit the growth of CaO_x crystals. This property of plants may be important in preventing kidney stone formation[18], CaO_x crystals induced by urinary macromolecules was less tightly bound to epithelial cell surfaces, which are then excreted with urine[19]. *Pedalium murex* contains various chemical compounds[15], which themselves possess ability to inhibit the crystallization of calcium oxalate thereby preventing crystal growth and aggregation. Electron microscopic observation revealed that the extract visibly reduced the crystal size with significant decrease in population of CaO_x crystals. These results indicate that the seed extract of *P. murex* is highly responsible for the inhibition of CaO_x crystal growth. This could be beneficial in the prevention of stone formation. Not only will the formed crystals be smaller, but the implication that these crystals might have a lower likelihood of aggregation shows that the crystals formed could more easily be eliminated by natural means[20].

Medicinal plants are used for the prevention of kidney stones from a long time in different countries[21,22]. The

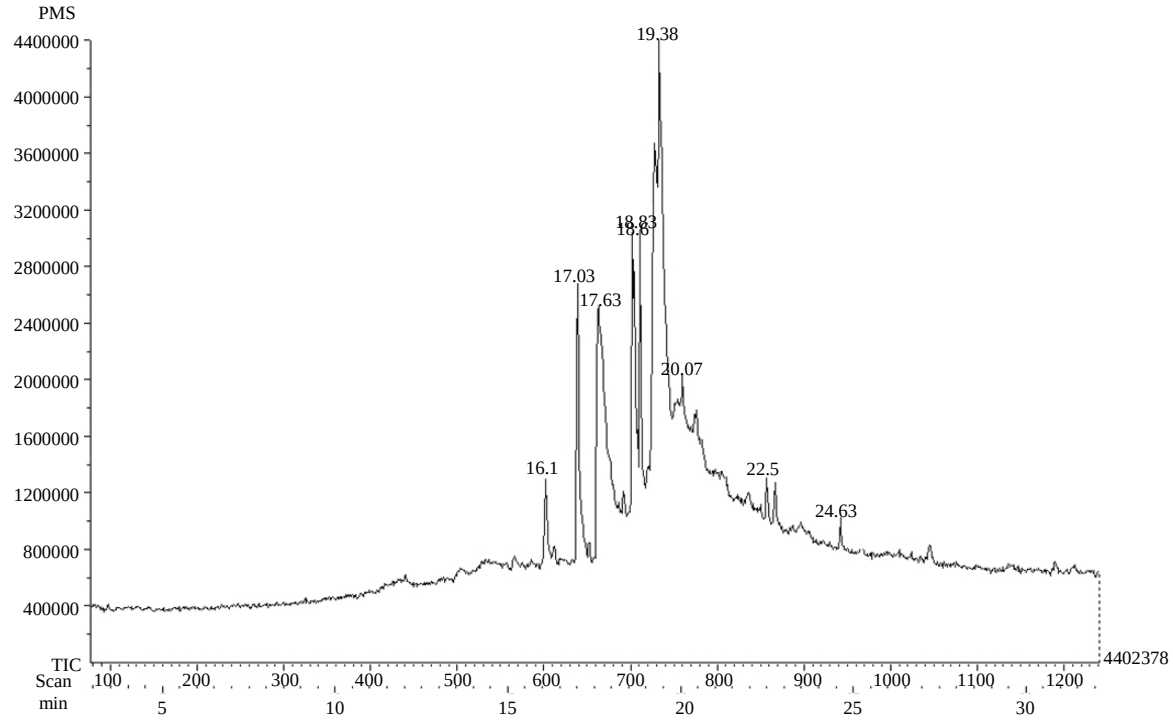

Fig. 3: GC-MS chromatogram of seeds of *P. murex*

Table 1: Compounds identified in the seeds *P. murex*

RT	Name of the compounds
24.63	But-2-endiamide, N,N',-bis(4-methoxy phenyl)-2
22.5	Coumarine,3-[2-(1-methyl-2-imidazollylthio)-1-oxoethyl)-
20.07	Phytol
19.38	Oleic acid
18.83	Cyclopentaneundecanoic acid, methyl ester
18.6	Quinoxaline,2-isopropyl-3-phenyl,4-oxide
17.63	Estra-1,3,5(10)-trien-17a'-ol
17.03	Tridecanoic acid, methyl ester
16.1	3-Cyclohexen -1-ol-,4-methyl-(1-methylethyl)-

preventive efficiency of phytochemicals have been evaluated in kidney stones[23]. Totally, 9 compounds identified from the GC-MS study (Table 1 and Fig. 3). GC-MS study revealed the presence of oleic acid (classified as a monounsaturated omega-9 fatty acid), phytol, phytosterol, coumarine derivatives etc., (Table 1) may possesses a protective effect against development of calcium stones in the kidneys. This is very beneficial for stone formers since studies suggest that the biochemical pathways of a stone former will always promote stone formation but the presence of growth modifiers such as phytochemicals found in seeds of *P. murex* could aid in the elimination of stone.

CONCLUSION

It is concluded that Scanning Electron Microscopy (SEM) is a necessary tool to identify and study several renal structures and their behavior as a consequence of the calculi removal, so that it is possible to supply important information for the surgical ways of intervention in order to prevent a relapse. The *P. murex* seed extract decreased the crystal size and inhibited crystal aggregation. The seed contains good amount of fatty acid and several phytochemicals. This study may suggest that *P. murex* can be used as therapeutic agent for the treatment of urinary calculi or its prevention.

SIGNIFICANT STATEMENT

Kidney stone disease is a common chronic disorder seen in humans and the most common type of renal stone is made of calcium oxalate. Kidney stones cause serious health problems such as severe pain, urinary tract obstruction and infection that adversely affect comfort of individuals. *Pedalium murex* Linn. is an important drug widely used in India for the patients of urolithiasis. Scanning Electron Microscopy (SEM) can provide degradation/dissolution information of urinary calculi. Common methods of kidney stone analysis, such as light microscopy and FTIR, provide limited information of the stones. SEM gives high spatial resolution (~1 nm). SEM can provide degradation/dissolution information of urinary calculi. This study may suggest that *Pedalium murex* seeds and its phytochemicals can be used as therapeutic agent for the treatment of urinary

stones or their prevention. This investigation will helpful in developing new drugs for the treatment of kidney stones, with therapeutic and economical value in the future.

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