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Evaluation of anti-urolithiatic potential of ethyl acetate extract of *Pedalium murex* L. on struvite crystal (kidney stone)

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ABSTRACT

Pedalium murex (L.) is a traditional herb, commonly used for the treatment of kidney stone related problems. Struvite stone can swiftly grow and become 'staghorn calculi' in kidney and its associated areas, which is the most aching urological disorder. The present study investigated the anti-urolithiasis activities of ethyl acetate extract of *P. murex* L. (EAEP) against struvite crystal. The antibacterial activity of EAEP examined against several urease producing bacteria. It showed the minimum bactericidal concentration (MBC) against *Escherichia coli* and *Staphylococcus aureus* (>125). On the other hand, total mass, volume, number, growth rate and dissolution rate of synthesised struvite crystals were observed at different concentrations 0.5%, 0.75%, 1% of EAEP and without EAEP. In which, EAEP addition showed appreciably reduced struvite crystal. Alternatively, MgO (300 mg of EAEP/kg/body weight) induced urolithiasis of Wistar albino rat at the rate of 1 ml for 28 days. Various biochemical parameters in serum, urine and histological analysis of kidney were taken for evaluation. Significant results ($p < 0.05$) were observed in 1% EAEP (300 mg) treated group than cystone treated group. From the histological study, reduced renal damage and glomerular development were observed. Our experiment, *P. murex* L. enhances the reducing activity on struvite crystal and prevents the crystal formation both *in-vitro* and *in-vivo*. It can be suggesting that *P. murex* L. and its phyto-components could be used as remedy for the management of kidney stone by dissolving the struvite stone in kidney.

1. Introduction

Traditional medication is an essential source of potentially valuable compounds for the development of potential therapeutic agents.[1] A broad range of medicinal plant extracts are used as a raw drugs[2] and are known to be an essential supply of anti-infective compound with potential healing effects.[3] Moreover, this led to the invention of a novel drug against diverse diseases for the human being because of safe and efficient.[4] According to the information of the world health organisation (WHO), as many as 80% of the world's population expect their primary health care[5] because it is reliable, simple, nontoxic, eco-friendly and low adverse effect, which enhances its acceptability to consuming people.[6]

In the present work we look out the problem of most incurable disease such as kidney diseases around all over the world. Urolithiasis is a common disorder with multi-factorial formation by the combined influence of epidemiological, biochemical and genetic risk factors[7] and it is termed as stones which are located in any place within the urinary tract. The people with kidney stone in the US as 12% of males and 6% of females and in India also has 12% get affected[8,9] but it is approximately 12% of the world population get renal stone disease, with a reappearance rate of 70–81% of males and 47–60% of females.[10,11] Nowadays, young age people are also affected by the kidney stone and this will change their life style and

Abbreviations: EAEP, Ethyl acetate extract of *Pedalium murex* L.; MgO, Magnesium Oxide; MBC, Minimum Bactericidal Concentration.

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diet.[12] High incidence of urolithiasis distributed across the world are Scandinavian countries, Mediterrenanian, British Isles, Northern Australia, Central Europe, parts of Malaysia, China, Pakistan, India, Sudan, Saudi Arabia, UAE, Myanmar, Thailand, Indonesia and Philippines.[13] Usually, the urinary stones are crystal components such as brushite [$CaHPO_4.2H_2O$], struvite [$(NH_4)MgPO_4$- $6(H_2O)$], calcium oxalate [$Ca(COO)_2$], hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], etc. Among phosphates, struvite stone made of magnesium ammonium phosphate hexahydrate (MAPH; $MgNH_4PO_4 \cdot 6H_2O$) accounts 10–20% consider as the second predominant crystalline component which is rapidly growing and lifetime frightening disorder, especially in women.[14,15] Bacterial infection may stimulate struvite stone development by crystal adherence and are caused by the action of bacteria on urine[16] due to the alkalization of urine by the urease producing bacteria such as *Proteus* species, *Klebsiella*, *Pseudomonas* and *Corynebacterium* species also by *E. coli*.[17]

Struvite stone is mainly formed when the urinary tract is infected by urease positive microorganisms, especially *Proteus* sp which is the primary urease segregating bacteria in human[18,19] which is the enzyme, splits urea $(NH_2)2CO$, a natural element of urine and converted into ammonia (NH_3) and carbon dioxide (CO_2).[18] This will convert urine into alkaline nature and increase the concentration of NH_4^+, CO_3^{2-} and PO_4^{3-} ions along with Mg^{2+} favour the formation of crystals in urine.[20] Struvite stone does not exhibit any indications like other stones, frequent infection may be a symptom of struvite stone, mild pain and blood in urine may be seen. Moreover chronic infection can cause significant renal damage and if untreated can lead to end-stage renal disease (ESRD).[21,22] So it can be treated with antibiotics but it become more resistant to antibiotics[23] and there is a need to investigate different compound with antimicrobial property. From the traditional system, like ayurveda numerous drugs are derived for the treatment of diseases beyond this there is no well established through efficient pharmacological and clinical studies to be expecting for some herbal drugs and plants. Moreover these herbal drugs have effective in curative and preventing the repetition rate of renal calculi with no side effects.[24,7]

Pedalium murex (L.) is a small herb called in vernacular name as Yaanai Nerinji and it can be used for the treatment of puerperal diseases, digestive tonics, ulcers, fevers, wounds, aphrodisiac, antitussive, appetizer properties and useful in vesical calculi, urinary discharge, gonorrhea etc.[25–27] Fruits of this plant can be used for the treatment of genito-urinary disorders, infertility, impotency, intestinal colic, diabetes. A few researches have been done for its antimicrobial, hypolipidemic, aphrodisiac, nephroprotective, and anti-inflammatory activities.[26–28] It contains several flavonoids and alkaloids like pedalitin, diosmetin, dinatin, pedalin dinatin-7-glucuronide,[27] aromatic oil, tannins, glycosides, sterol and some nitrates, fat, resin, and gum, Caffeic acid, cumaric acid, daucosterol, ferulic acid, hepatatriacontonic acid, vanillic acid,[27] ursolic acid and sitosterol and it has different pharmacological activities. Though it is used as a folk medicine for renal stone, its effectiveness and mechanism as the anti-urolithiatic agent are still unknown. Therefore, in the present investigation is intended to explore the medicinal property of *P. murex* L. plant, using ethyl acetate extract of whole plant.

2. Materials and methods

2.1. Identification, collection and extraction of plant material

The whole plants of *P. murex* (L.) were collected from Thanjavur district, Tamil Nadu, India. It was identified taxonomically by Rev Dr. S. John Britto SJ, Director, The Rapinat Herbarium and Centre for Molecular Systematic, St. Joseph College (Autonomous), Tiruchirapalli, Tamil Nadu, India and the voucher number is RHPM SR 001. The freshly dried whole plants of *P. murex* (L.) was powdered by an electrical blender and passed through 20 m mesh filter for the coarse powder. The powdered material was extracted with 1:2 w/v of ethyl acetate using Soxhlet apparatus. The extract was concentrated at 45 °C using rotary vacuum evaporator under reduced pressure and evaporated on a water bath until it appearance a thick paste.[29] It was kept in a refrigerator at 4 °C in an air tight glass bottle for the complete study. The yield was 15.1% w/w in terms of dried starting material.

2.2. Preliminary phytochemical study

Phytoconstituents of EAEP was qualitatively analysed using standard methods by Harborne, (1973).[30] It revealed the presence of steroid, glycosides, saponins, resin's, carbohydrates, amino acids, fixed oils & fats, gum & mucilage, quinone, anthraquinone, coumarins, flavonoids, tannins, alkaloids, terpenoids, phenolic compounds.

2.3. Test bacterial pathogens

Escherichia coli MTCC 433, *Staphylococcus aureus* NCIM 5021, *Proteus mirabilis,* MTCC 425, *Klebsiella pneumoniae* MTCC 432, *Pseudomonas aeruginosa* NCIM 5029 were taken for the experiment.

2.4. In–vitro assessment of antibacterial activity and MIC and MBC of EAEP

To determine antibacterial activity of EAEP were tested against positive and negative controls by disc diffusion method as described by Bauer *et al.*,.[31]

MICs were carrying out for EAEP (that show $\geq$ 8 mm diameter growth zone of inhibition in disc diffusion method) in 96-well micro-plates. The 1 mg/ml concentration of EAEP was serially diluted in 150 µl sterile Mueller-Hinton broth and each well was filled with 50 µl of bacterial culture (1×10^5 CFU/mL) and one well was kept as control for each test organisms, the micro-plate was covered and incubated at 37 °C at overnight. After incubation OD values were taken in microtiter plate reader at 600 nm[32]. The MBC was determined by sub-culturing the test dilution (used in MIC) on to a fresh solid medium and incubated further for 24 h. The concentration of plant extract that completely killed the organisms was considered as MBC.[32]

2.5. In–vitro study - single diffusion gel growth technique

The modified single diffusion gel growth technique was employed to study the growth and inhibitory effect of struvite crystal using different concentration of ethyl acetate extract of *P. murex* L. extracts.[14] Sodium metasilicate (SMS) – [Na_2 Si O_3, $9H_2O$] solution (specific gravity 1.05) was used to prepare the gel. An appropriate amount of aqueous solution of 0.5 M ammonium dihydrogen phosphate (ADP) – [NH_4 H_2 PO_4, $2H_2O$] was mixed with the SMS and it was adjusted to pH 7.0. Then it was transferred into autoclaved test tubes (140 mm length and 25 mm diameter) to set the gel formation. All the test tubes and glass wares were autoclaved at 120 °C for 15 min for forming the silica hydro gel. After gelation was obtained, gently pour 20 ml supernatant solution of pure 1.0 M magnesium acetate – {$C_4H_6MgO_4.4H_2O$} (Control solution-without plant extract) and 20 ml of 1.0 M magnesium acetate was prepared with plant extract at 0.5%, 0.75% and 1.0% concentrations of ethyl acetate extract of *P. murex* L. (EAEP) on the set gels in the respective test tubes. After that the test tubes were

capped with airtight stopples. This entire experiment was carried out at the room temperature and it was also done in laminar flow chamber for maintaining the sterilised condition.

The following reaction was anticipated to take place in the gelation medium (Fig. 1):

$$NH_4H_2PO_4.2H_2O + (CH3COO)_2\,Mg\,.4H_2O \rightarrow NH_4MgPO_4.6H_2O + 2CH_3COOH$$

In the test tubes, noticeable growing/dissolving struvite crystal lengths were measured from the gel liquid interface (Fig. 2). After the completion of experiment, the crystals were removed from the respective test tube at a particular period. Subsequently, the crystal growth was measured.

2.6. Oral acute toxicity

For animal studies, Wister albino male rats (weighing between 180 – 200 gm) were collected from the Animal house, School of Pharmacy, Al-Shifa College of Pharmacy Perinthalmanna, Kerala, India. The animals were acclimatised to the laboratory condition in the metabolic cages and they were fed with water and standard animal pellet *ad libitum*. The animal facility was well ventilated and maintained at ambient temperature (28 ± 2 °C; 50–60% relative humidity) with 12 h light and dark cycles.

For testing of toxicological effects, we prepared different concentrations of EAEP which were fed orally (20, 50, 100, 200, 300, 500, 1000 and 2000 mg of EAEP/kg dissolved in water) and water was used as a control for all groups. All the animals were observed intensively for 14 days to assess their toxicity or mortality rate. The toxicity studies were carried out as per OECD guidelines (No: 423).[25] The experimental protocol was approved by Institutional Animal Ethics Committee constituted Guidelines (1195/Re/S/08/CPCSEA dt 01/07/2016).

2.7. Ethyl acetate induced urolithiasis model in rats

The animals were randomly divided into six groups containing six animals in each group and incubated for 30 days. Group 1: Control group were received standard pellet and drinking water. Group 2: Positive group were received standard drug cystone (500 mg/kg body weight) with 0.4% magnesium oxide in drinking water from 1st day to 30 days at the rate of 1.0 ml/rat/day. Group 3:

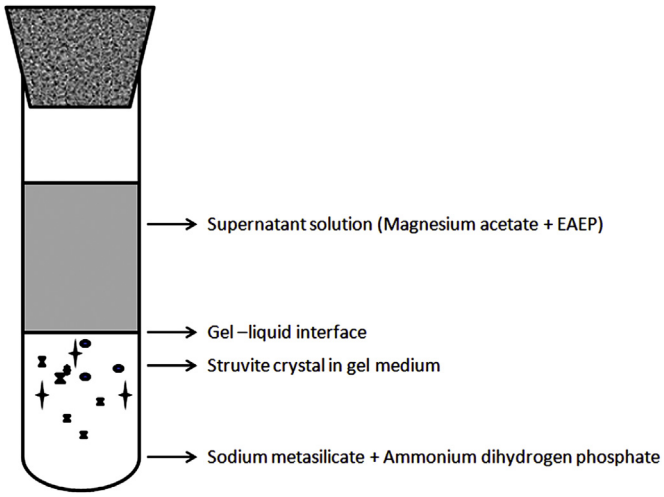

Fig. 1. Diagrammatic representation of single diffusion gel growth technique.

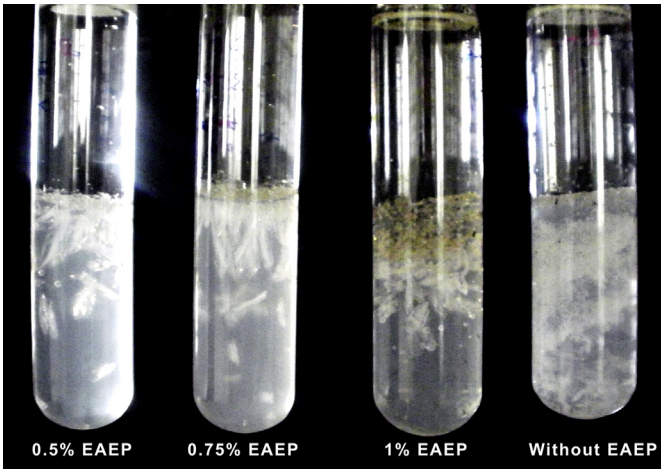

Fig. 2. Development of struvite crystals in gel medium with different concentration of EAEP.

Negative group were received with magnesium oxide (0.4%) in drinking water for induction of kidney stone till 30th day of incubation period. Group 4: Rats treated with EAEP (300 mg/kg body weight) along with MgO (0.4%) by oral administration up to 30 days at the rate of 1.0 ml/rat/day. Group 5: Post-treatment group receive MgO (0.4%) in drinking water for 15 days for the induction of struvite stone in kidney then fed with EAEP (300 mg/kg body weight) by oral administration for another 15 days at the rate of 1.0 ml/rat/day. Group 6: Pre-treatment group received EAEP (300 mg/kg body weight) for first 15 days at the rate of 1.0 ml/rat/day, and for next 15 days it was fed with MgO (0.4%) along with drinking water.

2.7.1. Collection and analysis of urine sample

For the analysis of urine, the 24 h urine samples were collected for the days of 0, 5, 10, 15, 20, 25 and 30 from rats housed using individual metabolic cages. Before the collection of urine, the rats were provided with water but no feed. Urine was collected in 50 ml beaker wherein a drop of hydrochloric acid was added to the sample and kept at 4 °C. Then, total urinary excretion of creatinine, uric acid, sodium, potassium and calcium were measured by standard biochemical kits (Span Diagnostics, Surat, India) and remaining samples were used for microscopic analysis.

2.7.2. Collection of serum sample for analysis

For the analysis of serum sample, the blood was collected retro-orbitally at the day of 0, 15 and 30th days under mild anesthetic condition. Animals were sacrificed by cervical decapitation for histological studies. Haematological parameters such as haemoglobin content (Qualigens Diagnostics), total erythrocyte and leukocyte count[33] were analysed in the blood. Serum samples were separated from the blood by centrifugation at 10 min at 10,000 rpm and evaluated for creatinine, uric acid, sodium, potassium and calcium by standard biochemical kits (Span Diagnostics, Surat, India). Remaining serum was diluted in the ratio of 1:10 with normal saline and kept it for further studies.

2.7.3. Microscopic analysis of urine

A fresh urine samples were collected from each group of each animal for crystalluria analysis without adding any preservatives. For microscopy of crystal deposition, 1 ml of the fresh urine sample was centrifuged at 3000 rpm for 10 min, and then discarded the supernatant. Then the sediment was kept in a slide with a cover slip. From this slide, type and number of crystals were identified

using the light microscope.

2.7.4. Histological assays

Histological assay of the kidney was followed by the method of Divakar *et al.*, (2010).[35] The sacrificed rat abdomen was incised and opened, and both kidneys were removed from each animal. Isolated kidneys were cleaned off extraneous tissue, weighed and rinsed with ice-cold normal saline. The kidney tissue pieces were taken from the each group and it was fixed by neutral formalin (10%) and afterwards embedded in paraffin film. The sections (5 μm thick) of kidney were stained with hematoxylin and eosin to study the alteration of histological changes and crystal deposition.

2.7.5. Statistical analysis

The data were expressed as arithmetic mean ± standard error of mean (SEM) of six rats (n = 6) and were analysed using analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. The difference was considered statistically significant at $P < 0.05$.

3. Results

3.1. In-vitro experiment

3.1.1. Antibacterial activity and assessment of MIC and MBC of EAEP

The antibacterial activity was carried out in EAEP by disc diffusion method (Table 1). EAEP showed moderately significant antibacterial activity against *E. coli* (21.6 ± 0.35) and *S. aureus* (17.1 ± 0.05), when compared with standard drug ampicillin (18.06 ± 0.03; 27 ± 0.00) and penicillin (18.03 ± 0.12; 29.06 ± 0.03) respectively. Subsequently, EAEP showed the MIC values of 615, 62.5, 125, 120 and 126 against the *E. coli*, *S. aureus, P. mirabilis*, *K. pneumoniae*, *P. aeruginosa* respectively and MBC values of EAEP against *E. coli and S. aureus* (>125). It revealed that EAEP has significant activity against pathogenic bacteria and also all selected pathogens were sensitive to EAEP.

3.1.2. Morphology and growth rate of crystal

Using gel growth technique, nutrients are continually provided for the crystal growth from the reactants. The dissolution rate is to be ensured for the preferred solutions. Among morphologically diverse struvite crystals (such as the dendritic type, prismatic type, rectangular platelet type and needle type), the dendritic type was grown in gel liquid interface. It was developed and observed in the test tubes. With and without plant extract test tubes were analysed the crystal as clear to translucent diaphaneity and it may also showed dark brown colorization because of insertion of the extract in the crystals. Fig. 3 showed the grown struvite crystal at 0.5%, 0.75% and 1% of EAEP and without extract gel medium. The concentration of plant extract was increased in set gel tubes, due to that the average lengths of the struvite crystals were decreased

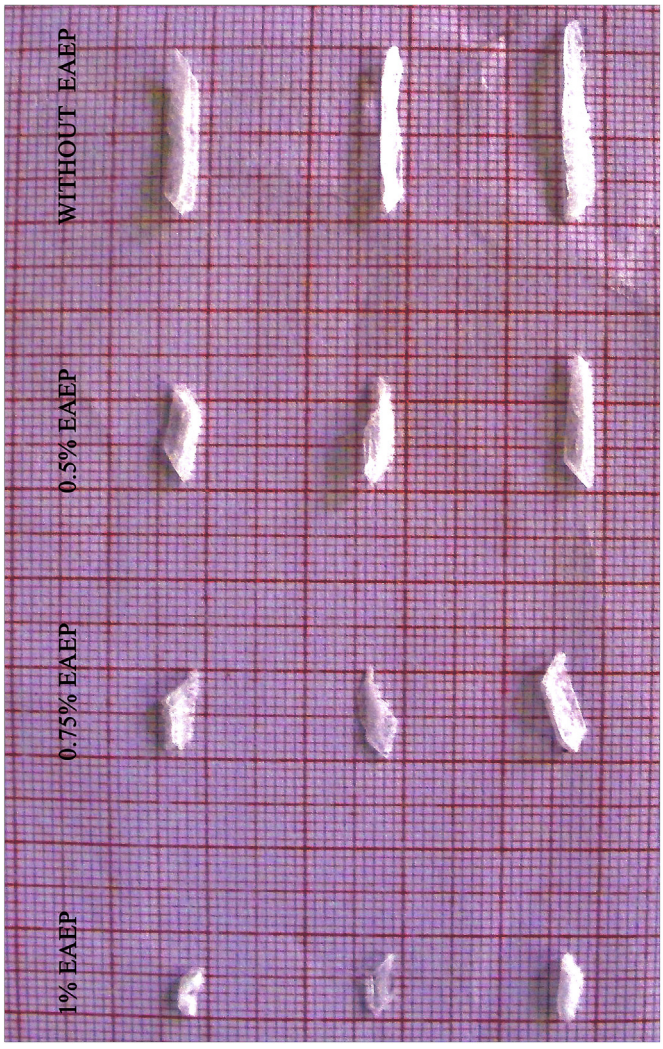

Fig. 3. Effect of different concentration of EAEP on struvite crystal growth.

(Fig. 3). Table 2 showed the inhibitory effect of EAEP on struvite crystal growth rate for five days with different concentration of extract, afterwards the dissolution was started. The average length of dendritic crystals were 1.3 cm in the without inhibitor gel tube, where as decreased length crystals were 0.7 cm, 0.6 cm and 0.5 cm in the presence of EAEP at 0.5%, 0.75% and 1% respectively. The growth rate of struvite crystal was decreased when the concentration of plant extract increased. The maximum growth was found in the gel tube for five days (1.22 cm, 1 cm, 0.82 cm and 1.52 for 0.5%, 0.75%, 1% and without inhibitor respectively). Subsequently, struvite crystal growth was reduced significantly in EAEP

Table 1
Assessment of antibacterial activity of EAEP against selected bacterial strains.

Pathogens	Antibacterial activity in mg/ml (Mean ± S.D)								
	Ethyl acetate extract			Penicillin			Ampicillin		
	Zone of Inhibition	MIC	MBC	Zone of Inhibition	MIC	MBC	Zone of Inhibition	MIC	MBC
E. coli	21.6 ± 0.35	615	>125	18.06 ± 0.03	0.39	>1.56	18.03 ± 0.12	0.39	>1.56
K. pneumoniae	15.1 ± 0.05	120	–	19.2 ± 0.1	6.25	>12.5	15.07 ± 0.06	6.25	>12.5
P. mirabilis	16.03 ± 0.03	125	–	18.1 ± 0.05	1.56	>3.125	12.1 ± 0.01	1.56	>3.125
P. aeruginosa	14.06 ± 0.03	126	–	15.2 ± 0.08	1.57	>3.126	14.1 ± 0.03	1.58	>3.126
S. aureus	17.1 ± 0.05	62.5	>125	27 ± 0.00	12.5	>25	29.06 ± 0.03	12.5	>25

(–) No activity.

Table 2
Growth rates of the Struvite crystals growing in the gel at gel–liquid interface for the different concentrations ethyl acetate extract of *Pedalium murex* (L.) at the end from 1st to 5th day. (Values in cm).

Number of days	Ethyl acetate extract of *P. murex*			Without inhibitor
	0.5%	0.75%	1%	
1	1.22	1.0	0.82	1.52
2	0.71	0.62	0.58	1.30
3	0.24	0.21	Dissolution starts	0.95
4	0.17	Dissolution starts	–	0.68
5	Dissolution starts	–	–	0.53

supernatant presented gel tube. The inhibitory effect of extract was clearly explained at the end of 1st day and the gel-liquid interface was 26.2%, 21.6%, 19.8%, 32.4% and at the 2nd day was 21.5%, 18.7%, 17.5%, 42.3% for 0.5%, 0.75%, 1% of EAEP and without inhibitor gel tube respectively. These results were demonstrating evidently the effect of plant extract on crystal growth.

3.1.3. Dissolution of struvite crystals

Fig. 4 showed the growth and dissolution of average length of the dendritic type struvite crystals at different days of experiment in the gel-liquid interface. The grown crystals were completely dissolved at 40–45 days in the EAEP mixed gel tube and in without inhibitor tube were showed some extent reduced level of crystals. The dissolution was increased significantly in different concentration of EAEP mixed tube than the without inhibitor tube. After the 5th day of incubation, the length of crystals was successively reduced. The dissolution rate of grown struvite crystal in the gel at gel liquid interface was lower in control and different concentration of EAEP at 0.5%, 0.75% and 1% at the rate of 1.5×10^{-2} cm/day, 3.9×10^{-2} cm/day and 6.6×10^{-2} cm/day respectively (Table 3). This report showed that the higher concentration of extract enhance the dissolution rate at 1% than 0.75% and 0.5% (53.3%, 26.6% and 13.3%). The result of without inhibitor gel tube dissolution was 6.6% only.

The growing crystals were increased from 0^{th} day to 5th day in the gel interface and followed by dissolution in progress. The average length of crystal increased significantly up to 10 days, then they decreased gradually and the depth of gel column increased from gel liquid interface. After 35th day, growing crystal length became stable in 0.5% concentration of EAEP mixed gel column. In 1% EAEP gel column result showed the reduced size of crystal than the 0.5 and 0.75% EAEP mixed gel. Fig. 5 a, b, c and d had proved these results directly, when the plant extract concentration increased in gel medium, the formation of the crystal and its length were decreased and dissolution rate was increased.

The fragmentation of crystal was attained due to the presence of plant extract, which may increase the fragment of the crystal from the fractured/cracked crystal. It can be noticed by the depth of fragmentation of grown crystal. Fig. 6 showed that 1% concentration of plant extract mixed gel-liquid interface was significantly higher than the other concentrations of 0.5% and 0.75%. The logarithmically increased level of fragmentation was seen in various concentration of plant extract mixed gel tube, which revealed that the concentration was increased; the depth of fragmentation of crystal was also increased. Fig. 7 showed that the mass and volume of the struvite crystals, which also proved that the increasing concentration of plant extract influenced on the total mass and volume of the Struvite crystal in the gel. Struvite crystal mass at 1% concentration of extract was 0.65 g and the volume was 0.33 cc and this result was almost twofold in without inhibitor gel crystal was

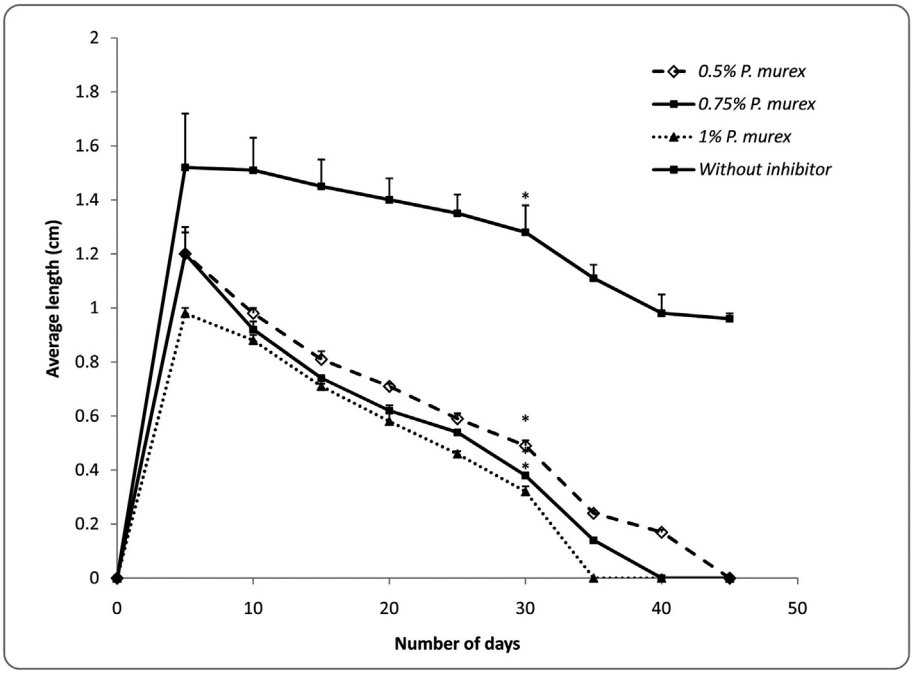

Fig. 4. Effect of EAEP on struvite crystal growth and dissolution in gel–liquid interface (*significant value at $P < 0.05$).

Table 3
Dissolution rates of the Struvite crystals in the gel at gel– liquid interface for the different concentrations.

Number of the supernatant solution with different concentration	Dissolution rate (cm/days)
0.5%	1.5×10^{-2}
0.75%	3.9×10^{-2}
1%	6.6×10^{-2}

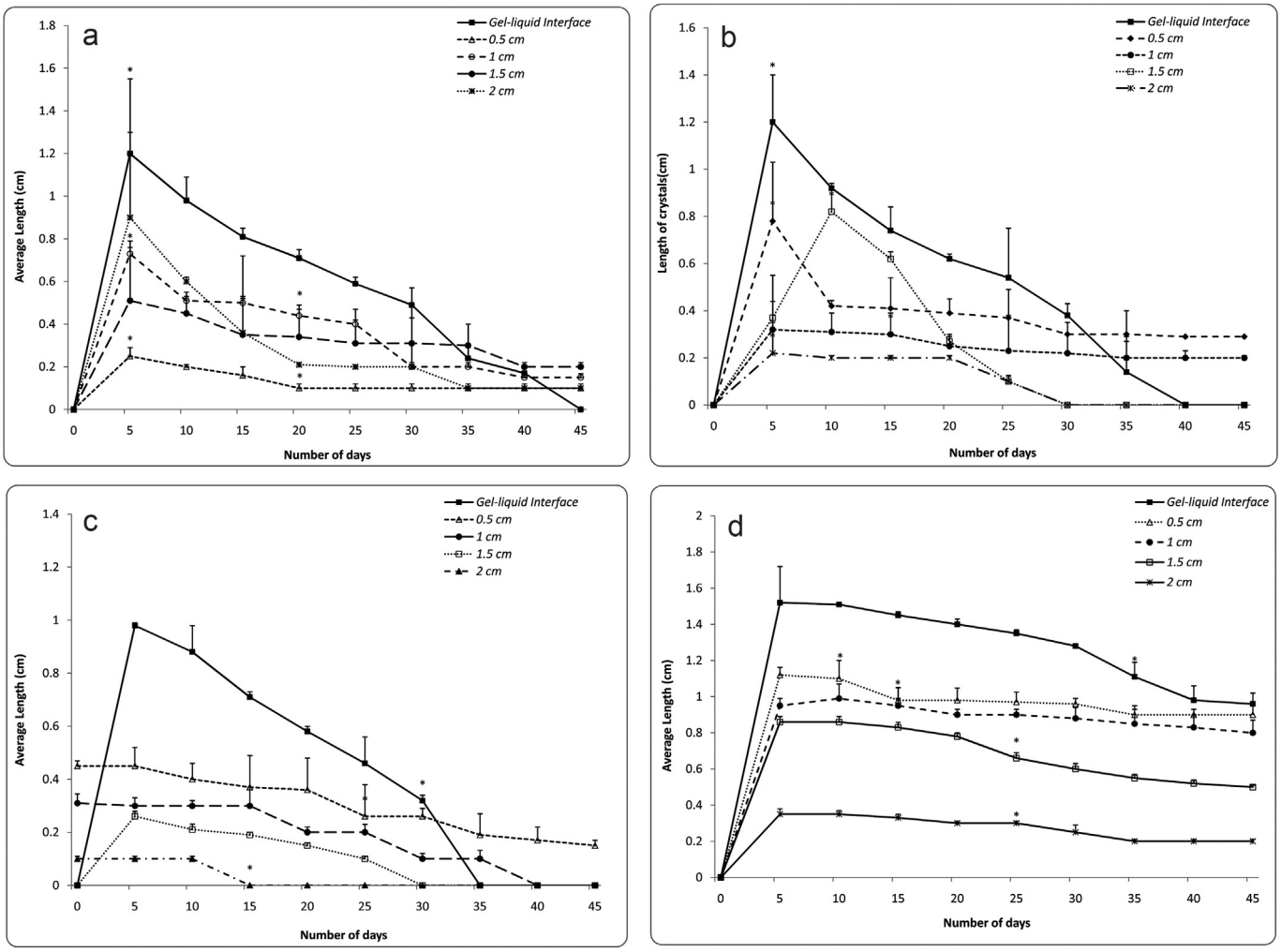

Fig. 5. Effect of EAEP on struvite crystal growth and dissolution at different depths from the gel–liquid interface (*significant value at $P < 0.05$). (a) Without inhibitor (b) 0.5% EAEP (c) 0.75% EAEP (d) 1% EAEP.

1.2 g and 0.68 cc. After fragmentation, below 1 mm length and 3 mm width of crystal was observed.

3.2. Effect of EAEP against struvite crystal using animal model

3.2.1. Biochemical parameters of urine sample

The urine analysis has been represented in the Fig. 8a and b. From the analysis, uric acid concentration was 2.9 mg/dl in control group and alternatively, the level was increased in the 0.4% magnesium oxide group as 3.8 mg/dl for the first 15 days and the concentration 2.8 mg/dl, 4.5 mg/dl for the next 30th day analysis. But there was a reduction in the concentration as 1.9 mg/dl and the level has been reduced in 30th day at the range of 1.6 mg/dl which received cystone as feed. There is significant reduction in group 4 animal of 0.8 mg/dl and also it was reduced to 0.6 mg/dl at the end of 30th day. In the post-treatment group 5 received first 15 days of magnesium oxide the uric acid level 1.8 mg/dl but in the next 15 days, which was received 1% ethyl acetate extract of plant *Pedalium murex* (L.) was decreased to the level of 1.4 mg/dl. In the pre-treatment group 6 there was a decreased level of uric acid from 2.6 mg/dl up to 1.8 mg/dl.

Then the sodium and potassium level of control group, group 2, group 3, group 4 for the first 15 days was in the range of 36.6 mg/dl and 132.3 mg/dl, 18.2 mg/dl and 53.6 mg/dl, 48.2 mg/dl and 201.8 mg/dl and 12.8 mg/dl and the level at the end of 30th day the concentration was 38.5 mg/dl, 15.2 mg/dl, 48.2 mg/dl and 10.2 mg/dl. In the post treatment and pre-treatment group also showed significant effect at the level of sodium due to the effect of plant extract. Then the calcium level also decreased in the group receiving plant extract when compared to the group 1 and group 2 and the level was 6.4 mg/dl to 6.2 mg/dl at 30 days of feeding. From the analysis, we concluded that the level of uric acid, sodium,

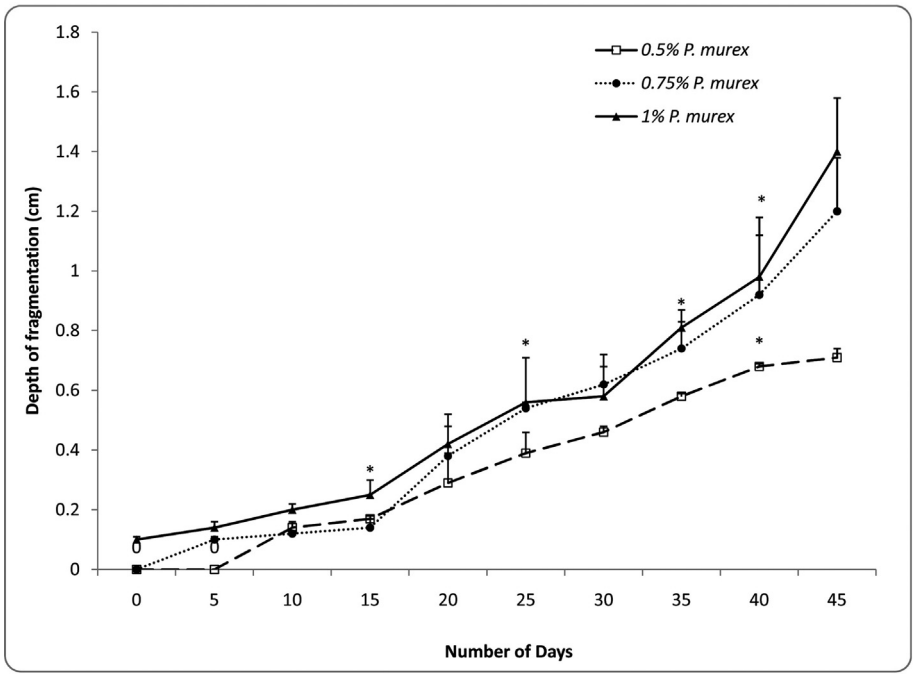

Fig. 6. Depth of fragmented struvite crystals in gel tube (*significant value at $P < 0.05$).

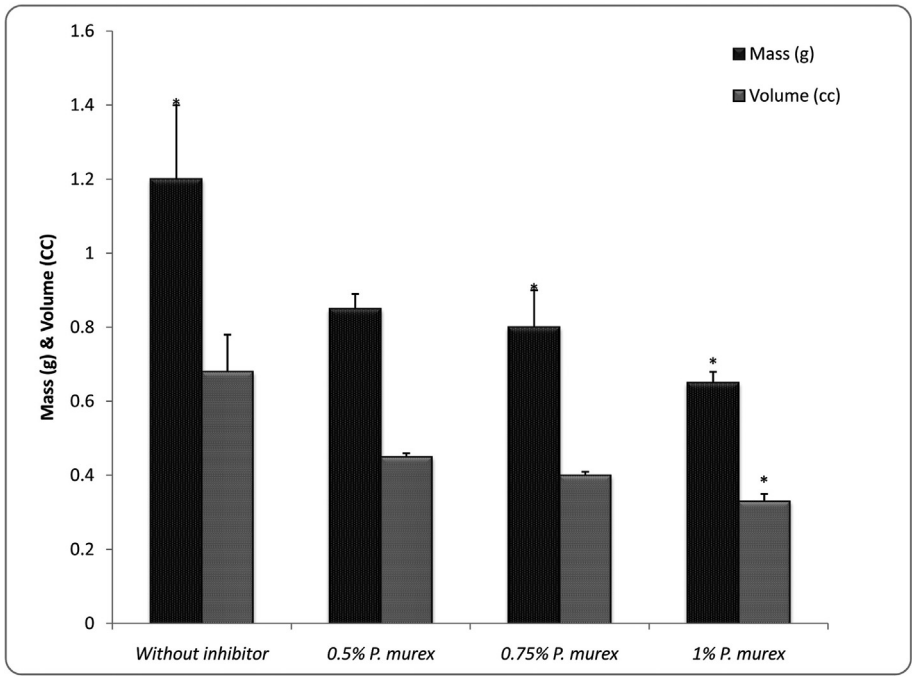

Fig. 7. Total mass and volume of struvite crystals at different supernatant solutions in gel tube (*significant value at $P < 0.05$).

potassium and calcium was significantly reduced in the group feeding with the 1% plant extract compared the control group 1and cystone group 2.

3.2.2. Biochemical parameters of serum sample

The serum analysis of magnesium oxide induced urolithiasis rat for the 15th and 30th days were showed in the Fig. 9a and b. Creatinine, uric acid, sodium, potassium and calcium were calculated. The level of creatinine in group 1 and group 2 are 7.42 mg/dl and 7.45 mg/dl respectively which indicate that there is no effect of cystone in crystal deposition. On 30th day in group 2, the level of creatinine was increased up to 8.8 mg/dl concentration and also be increased in group 3 from 8.49 mg/dl to 10.2 mg/dl. But in the group 4, there was a reduction from 6.24 mg/dl at 15th day to 6.21 mg/dl at 30th day of analysis because of the significant effect of inhibition of crystal deposition. The decreased level of creatinine was found in group 5 (6.8 mg/dl to 6.5 mg/dl) and group 6 (7.6 mg/dl to 7.1 mg/dl) when compared to the group 2.

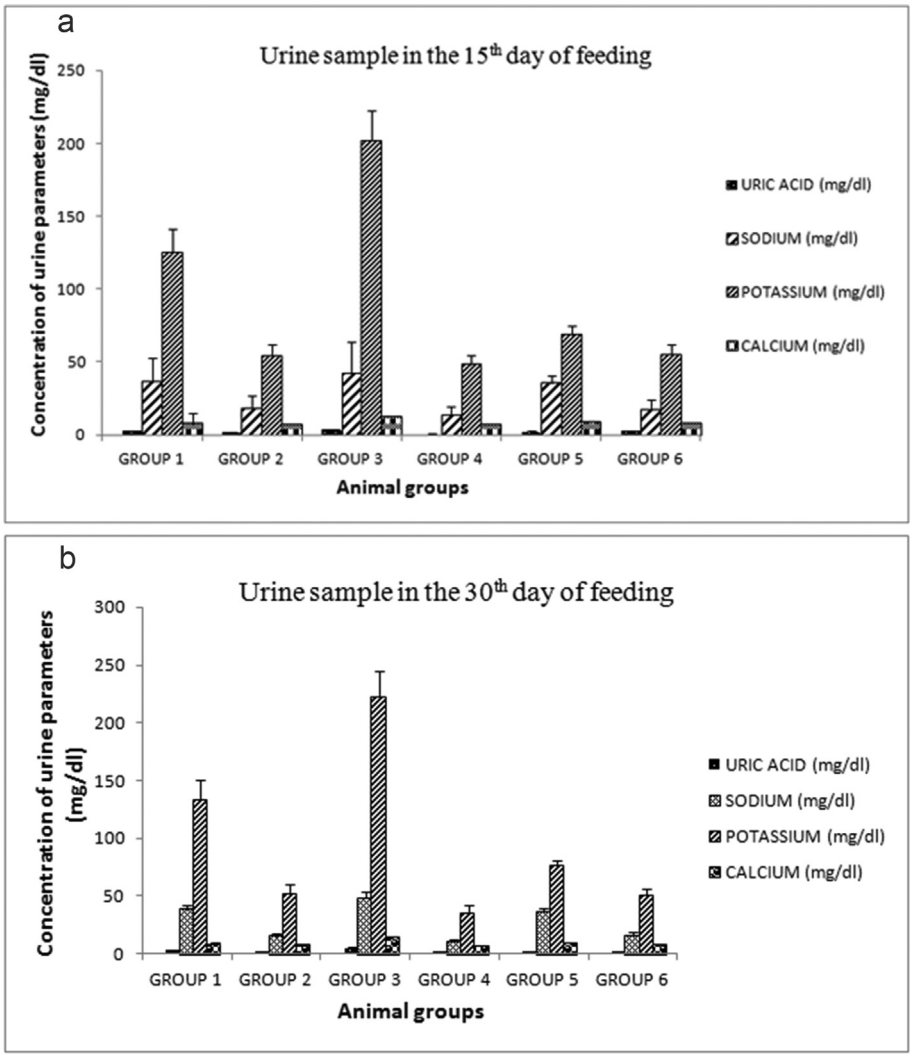

Fig. 8. a Biochemical parameters of urine sample at 15th day of feeding (*significant value at $P < 0.05$). b Biochemical parameters of urine sample at 30th day of feeding (*significant value at $P < 0.05$).

The level of uric acid in group 1 and group 2 was 2.1 mg/dl and 2.4 mg/dl respectively at 15th day of feeding, but it was increased up to 2.7 mg/dl and decreased in group 2 at the end of 30th day. In group 3, the uric acid level was increased from 3.1 mg/dl to 3.2 mg/dl. It may due to the sedimentation of crystals. But in group 4, the level of uric acid was decreased from 1.9 mg/dl at 15th day to 0.9 mg/dl at 30th day of analysis. The level of uric acid was reduced in the post-treatment and pre-treatment group due to the effect of plant extract in the induced urolithiasis rats.

The level of sodium in control group 1 was 38.2 mg/dl to the level 22.2 mg/dl at 30th day of analysis. In the group 2 was in the range of 16.8 mg/dl to the12.7 mg/dl at 30th day but decreased the level of sodium from 12.5 mg/dl to 8.2 mg/dl was found in group 4 when compared to group 1 and group 2. Sodium level was reduced in post treatment group 5 from 14.6 mg/dl to 9.6 mg/dl and pre-treatment group 6 from 36.3 mg/dl to 17.8 mg/dl. It may be due to the effect of plant extract on the induced urolithiasis rats. The level of potassium and calcium in group 1 and group 4 on 15th day (132.3 ± 18.8 and 34.8 ± 7.2) and 30th day (8.1 ± 1.2 and 6.2 ± 0.6) respectively decreased.

From over all analysis, the level of creatinine, uric acid, sodium, potassium and calcium were reduced in EAEP treated groups compared to control and cystone treated groups. This may be the effect of the presence of plant compounds on the reduction of crystals deposition in kidney and thus decrease the level of sodium potassium and calcium in the blood.

3.2.3. Haematological analysis of blood sample

In haematological analysis, total haemoglobin, total leucocytes and total erythrocyte count were significantly increased in all groups compared to control at 15th and 30th day of feeding (Table 4). The haemoglobin content was increased in group 2 and group 4 when compared to control. The leucocytes count also constantly increased in the group 4 and other groups. Similarly, the total erythrocytes count also increased in the group 4 compared to control. Based on the blood parameter, cell count was gradually increased when compared to the control group 1 and cystone group 2 (see Table 5).

3.2.4. Microscopic analysis of urine sample

The microscopic view of struvite crystals in urine sample (Fig. 10) showed that the presence of crystal in control (group 1), group 2 confirmed the presence of few struvite type crystals. In group 3, there was the presence of high amount of struvite crystals which were received 0.4% MgO. However, in group 4 and group 5, showed the noteworthy diminution of crystals and denatured

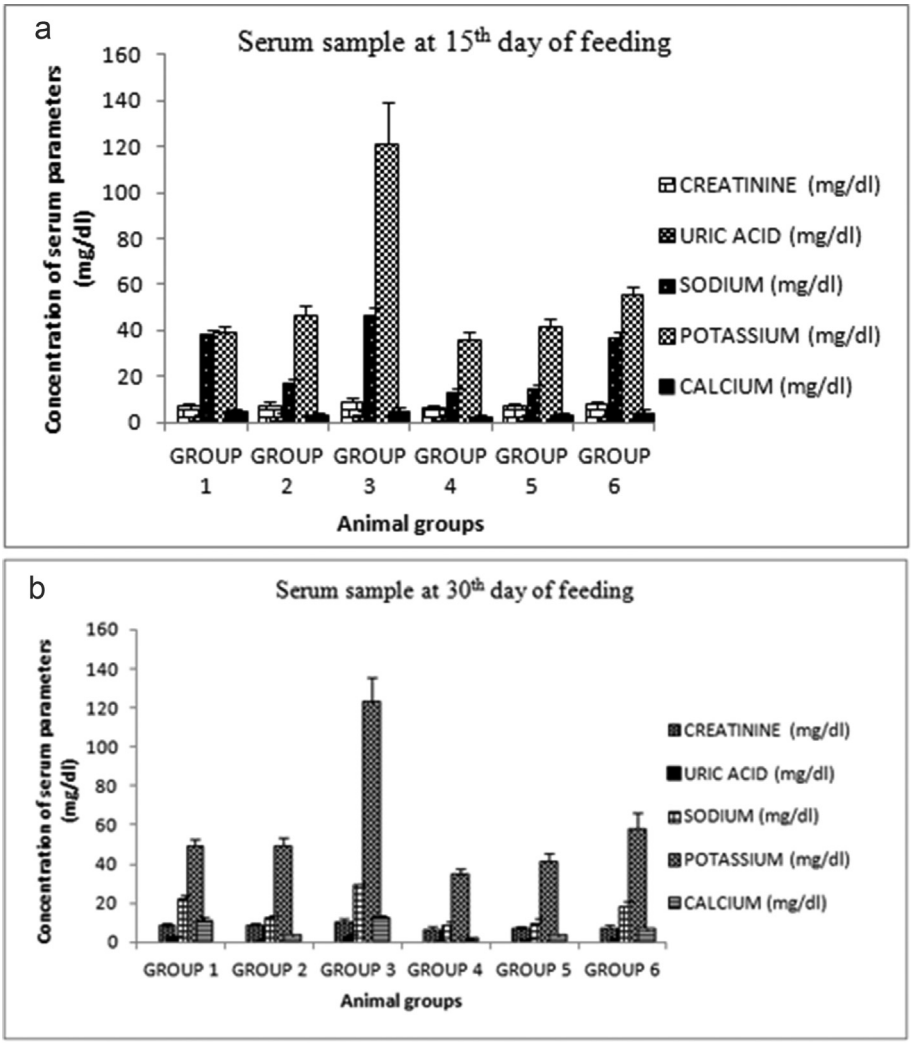

Fig. 9. a Biochemical parameters of serum sample at 15th day of feeding (*significant value at $P < 0.05$). b Biochemical parameters of serum sample at 30th day of feeding (*significant value at $P < 0.05$).

Table 4
Leucocytes count, erythrocytes count and haemoglobin count were obtained from 15th day and 30th day of feeding.

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
15th day of feeding						
Leucocytes count	544 ± 40.1	565 ± 42.1	551 ± 41.5	608 ± 42.3*	589 ± 39.3*	575 ± 38.3*
Erythrocytes count	6.8 ± 1.02	6.16 ± 1.23	5.18 ± 1.23	7.31 ± 1.21*	6.43 ± 0.9*	6.16 ± 1.1
Haemoglobin count	13.6 ± 1.31	13.9 ± 1.23*	11 ± 1.31	13.9 ± 1.3	13.7 ± 1.21*	13.5 ± 1.33
30th day of feeding						
Leucocytes count	556 ± 41.1	568 ± 42.3	565 ± 40.1	625 ± 38.3*	601 ± 39.1*	605 ± 37.3*
Erythrocytes count	6.6 ± 1.01	6.5 ± 1.03	4.9 ± 1	7.4 ± 1.3*	6.6 ± 1.2*	6.21 ± 1.02
Haemoglobin count	13.8 ± 1.32	13.7 ± 1.21	10.6 ± 1.22	14.2 ± 1.3*	13.9 ± 1.21	14.1 ± 1.23*

Values are expressed as mean ± SEM (n = 6). Hemoglobin (Hb: g/dl), Erythrocytes (RBC: 10^6 cell mm^3), Leucocytes (WBC: 10^4 mm^3) Statistical differences ($P < 0.05$) among groups are indicated by asterisk (*).

structure of struvite crystals when compared to positive and negative control group. In the pre-treatment group 6, the struvite crystals were decreased in number, compared to negative group 2 and group 3.

3.2.5. Histological analysis of kidney sample

The photomicrograph of kidney tissue section for 15th and 30th day of feeding was illustrated in Fig. 11 and Fig. 12. At 15th day, no variations were observed in organs of group 1 and group 4 and also small variation in glomerular observed in group 2. But, crystal deposition occurred in group 3 and showed some destruction in glomerular and tubular dilation. Crystal formation and slight alteration in glomerular and tubular destructions were found in group 5 and group 6. At 30th day, no changes were found in group 1, but slight changes in the tubular dilation were originated in cystone group 2. Group 3 showed completely damaged glomerular and tubular destruction. Group 5 and group 6 showed some deposition of crystal in the glomerular and tubular area, only thing is there was

Table 5
Total haemoglobin count, total leucocytes count and total erythrocytes count for 30th day of feeding.

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Total leucocytes count	556 ± 41.1	568 ± 42.3	565 ± 40.1	625 ± 38.3*	601 ± 39.1*	605 ± 37.3*
Total erythrocytes count	6.6 ± 1.01	6.5 ± 1.03	4.9 ± 1	7.4 ± 1.3*	6.6 ± 1.2*	6.21 ± 1.02
Total haemoglobin count	13.8 ± 1.32	13.7 ± 1.21	10.6 ± 1.22	14.2 ± 1.3*	13.9 ± 1.21	14.1 ± 1.23*
HCT (PCV)	35.6 ± 1.21	39.6 ± 1.23*	30.6 ± 1.16	37.2 ± 2.1*	34.8 ± 1.2	33.2 ± 1.9
MCV	5.4 ± 0.21	6.1 ± 0.32*	6.2 ± 0.1*	5.03 ± 0.1	5.3 ± 0.2	5.4 ± 0.3
MCH	2.1 ± 0.01	2.09 ± 0.2	2.2 ± 0.1*	1.9 ± 0.2	2.1 ± 0.2*	2.3 ± 0.3*
MCHC	0.39 ± 0.01	0.35 ± 0.02	0.35 ± 0.02	0.38 ± 0.02	0.39 ± 0.02	0.42 ± 0.01*

Values are expressed as mean ± SEM (n = 6). Hemoglobin (Hb: g/dl), Erythrocytes (RBC: 10^6 cell mm^3), Leucocytes (WBC: 10^4 mm^3) Statistical differences ($P < 0.05$) among groups are indicated by asterisk (*).

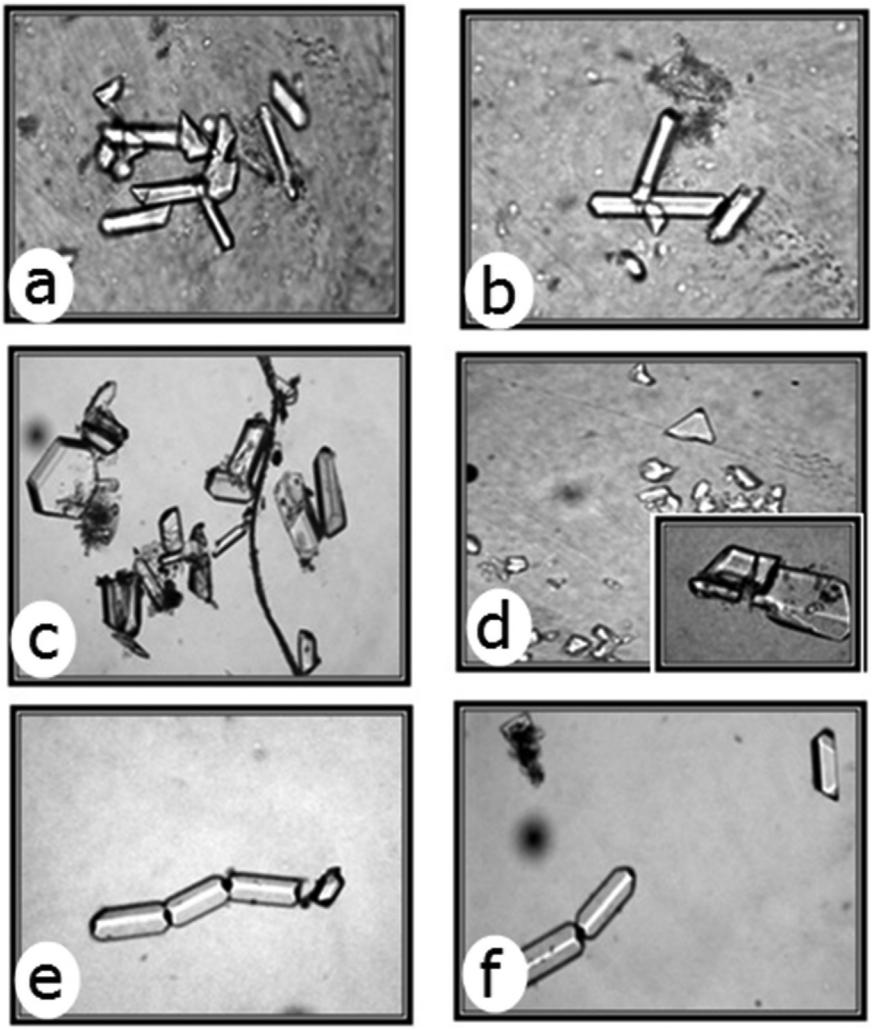

Fig. 10. Microscopic examination of urinary struvite crystals of experimental rats (at 40x magnification). (a) Group 1 (Control) (b) Group 2 (Positive control) (c) Group 3 (Negative control) (d) Group 4 (Normal Treatment) (e) Group 5 (Post-Treatment) (f) Group 6 (Pre-Treatment).

no damage or alteration in group 5 but in the group 6 some alteration were also found. In this histological study concludes that the EAEP treated group 4, no alteration were observed in the ultra structure of kidney. From this, during diseased condition, the plant extract play a major role to control/prevent the disease too.

4. Discussion

In the present investigation, we analysed the antibacterial resistant activity, anti-urolithiatic activity of ethyl acetate extract of the whole plant *P. murex* L. used in *in-vitro* and *in-vivo* rat model. From the testimony and knowledge of tribal people, the traditional medicine used for lithiasis activity and it can dissolving, expelling from urinary tract after few days of consumption.[36] In the current study, the animals were treated with EAEP for dissolving effect of struvite stone. By the increasing of antibiotic resistant microbes, it has to be need to find out the innovative drugs from medicinal plants particularly in pharma industries, which are widely available sources because it has no side effects, less expensive and have shown antimicrobial effects[37,38] and its therapeutic value known to

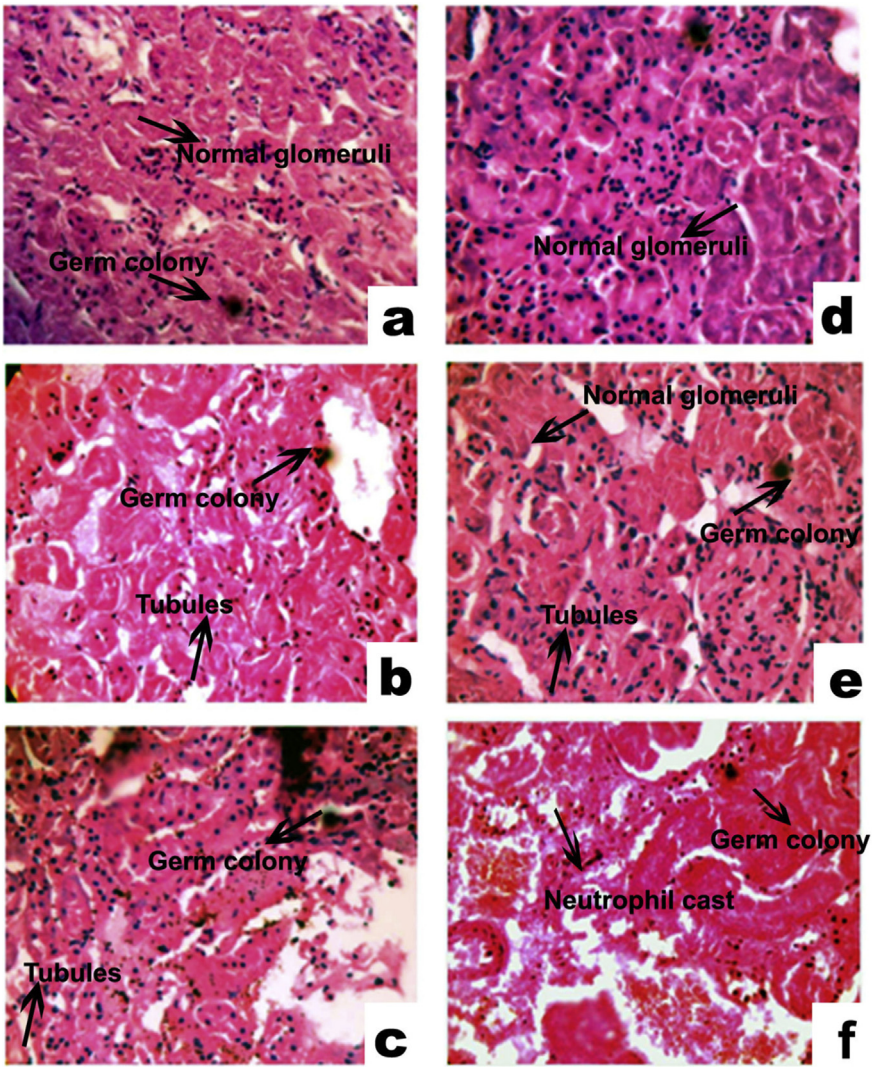

Fig. 11. Photomicrograph of kidney tissue section of experimental rats on 15th day of feeding (at 40x magnification). (a) Group 1 (Control) (b) Group 2 (Positive control) (c) Group 3 (Negative control) (d) Group 4 (Normal Treatment) (e) Group 5 (Post-Treatment) (f) Group 6 (Pre-Treatment).

all over the world for antibiotic development.[38] Pharmaceutical industries mainly focused on developing the antibiotic agents against pathogenic resistant strain in recent years.[39] The presence of a medicinally active compound in plants such as coumarins, flavonoids, glycosides, phenols, saponins, steroids and tannins are most responsible for a vital role in all diseases.

The bacterial isolates used in this study contain urinary pathogens such as *E. coli* and *P. mirabilis* is known to cause urinary tract infections. These microbes are susceptible to the plant extract which support to use as a traditional medicine for the effective treatment of urinary diseases caused by urinary tract infected (UTI) bacteria. From the analysis, the EAEP showed effective activity against these pathogens. It was compared to previous studies which have to be done in the different extract at different parts such as ethanolic extract of *P. murex* L. root proved as antimicrobial drugs against different pathogens.[40] From our findings, the EAEP was found to effective against UTI bacteria *E. coli*, *S. aureus, P. mirabilis*, *K. Pneumonia* and *P. aeruginosa* pathogen.

Kidney stone has different types including non–infection and infection stone because it originates from metabolic disturbances or through changes so far unknown and also with infections of urinary tract. UTI was mostly caused by gram-negative bacteria include *E. coli*, *Klebsiella*, *Enterobacter* and *Proteus* which also by gram positive *Staphylococcus* sp. play a role in the infection.[41] Bacterial infection can affects any part of urinary tract[42] if not treated pass through to the urethra and they produce end products of urealysis damage. It can cause infection not only to human but also animals and birds suffer from it. Mostly infection occurs in women than men due to the presence of shorter urethra.[43] The main causative factor for the stone formation by urinary supersaturation of different element present in the urine.[44,45] It may cause serious medical consequences such as extreme obstruction, hydronephrosis, infection and haemorrhage in the urinary tract system. The stone can be formed based on accumulation of minerals with four different stages includes nucleation, crystal growth, crystal aggregation and crystal retention. Struvite urolithiasis is a foremost illness in animals such as dogs, cats, rats and ruminants by urease-producing pathogens referred as infection stones.

We have analysed the inhibitory effect of EAEP by single diffusion method on the development of struvite stone. In earlier work, *in-vitro* inhibition of struvite crystal growth in artificial urine using acetohydroxamic acid (AHA) has been reported by Hamm *et al.*, (2000).[47] The AHA is chiefly acts as urease inhibitors, which may interrupt the struvite formation. By dietary regulations in cats, the

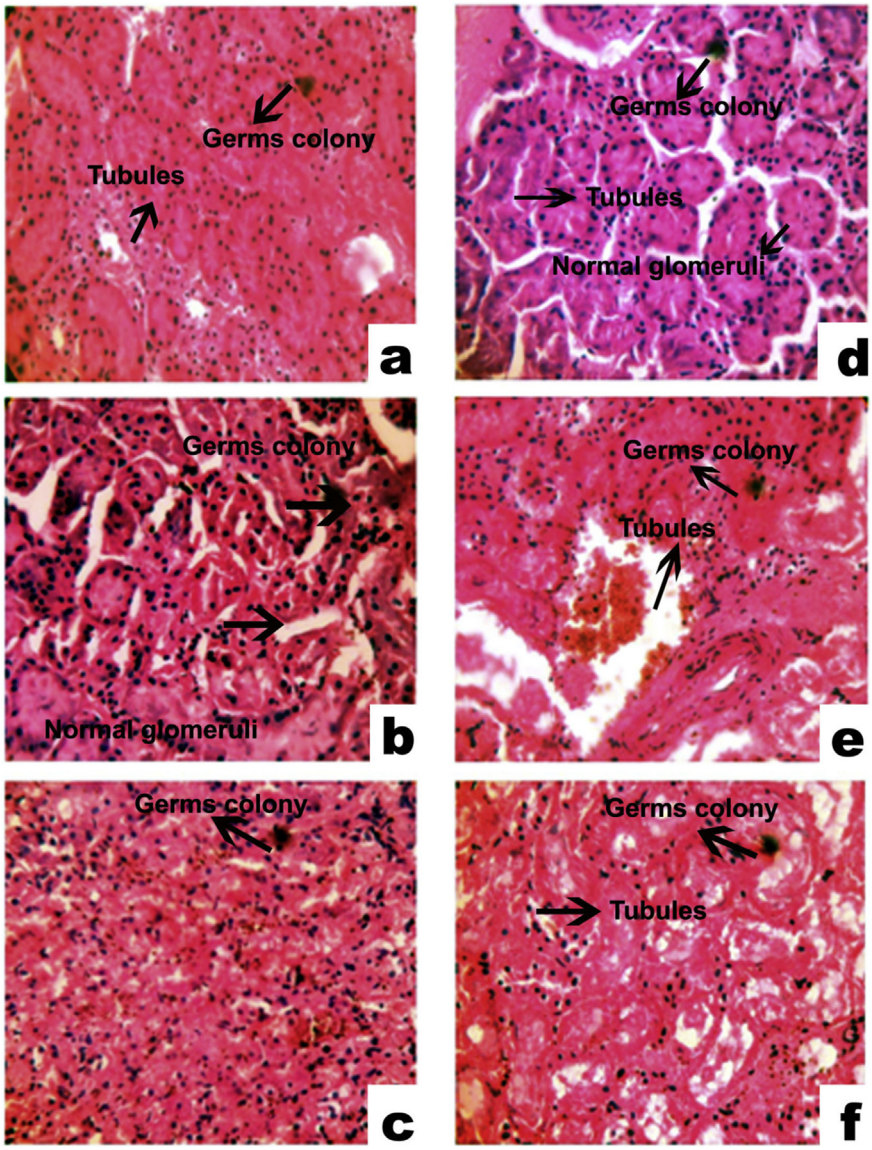

Fig. 12. Photomicrograph of kidney tissue section of experimental rats on 30th day of feeding (at 40x magnification). (a) Group 1 (Control) (b) Group 2 (Positive control) (c) Group 3 (Negative control) (d) Group 4 (Normal Treatment) (e) Group 5 (Post-Treatment) (f) Group 6 (Pre-Treatment).

risk of struvite formation can be minimized by slightly acidifying the urine pH. From the recent analysis, *Boerhaavia diffusa* extract,[14] *Citrus medica* juice,[48] *Rotula aquatica* (L.)[49] and *Commiphora wightii* extract[50] exhibited inhibitory effects on the struvite growth in *in-vitro* condition. Similarly, in our experiment, struvite crystal was inhibited and the length of crystal also considerably reduced by the plant *P. murex* L. mixed test tube.

In our results, the dimensions of fragmented crystals were between from 1 mm to 4 mm. These results were coincidently match with the result of Chauhan *et al.*, (2011)[49] who stated that the effect of *R. aquatica* L. root extract reduced average size of the crystal fragmentation was 1 mm and the dimension was 5 mm. This maximum size of the urinary calculi can easily run off from the urinary tract. The total mass and total volume of struvite crystals can be reduced in *in-vitro* condition using *P. murex* L. extract. An *in-vitro* study of growth inhibition of calculi was developed in the artificial urine by acetohydroxamic acid,[51] which acts on urease that disturbs the length of growing struvite crystal and crystal aggregation.

By the oral route, the traditional medicines are usually taken and by the same route were used for evaluation of anti-urolithiatic activity of *P. murex* L. against MgO induced renal calculi in rats. The variety of crystal formed in human can be predicted by the alteration of pH because the crystal formation is pH dependent and dissolution of stone can be done by variation in urinary pH. The uric acid type stone are likely formed means the pH is acidic 5.0 or lower but if it is 5.0–6.5 means calcium oxalate type can be formed and it can be alkaline (i.e. above 7) indicates magnesium ammonium phosphate are formed. In the urolithiasis animal study, the pH 6.0–7.0 to 7.5–8.5 was increased upon the formation of stones in the foreign body. This alkaline condition, urine can deposit with magnesium, which may composite with ammonium and phosphate excreted in urine. By the treatment with EAEP, it can be reduced the pH values to 6.0–7.5 that can reduce in stone weight and urinary flow of stone indicating the control of extract in dissolving urinary stone. This can be the evidence of the extract for the reduction of stone formation. Struvite stone is referred as triple phosphate stone because this stone can be combined with calcium,

magnesium, ammonium and phosphate moreover phosphate highly deposited urine due to alkaline condition because it is less soluble in this condition. Our experimental plant *P. murex* L. significantly reduced the stone in an animal model, it may be due to the action of chemical composition of the plant extract.

Consistent with previous reports, stone induction by MgO caused magnesium phosphate (Struvite) crystal and also excretion of magnesium in the group 1, 2 and 3. Calcium, magnesium, phosphate and uric acid were reduced by EAEP treated group 4, 5 and 6. Normal urine contains many inorganic and natural inhibitors of crystallization, magnesium is one such well known inhibitors. The magnesium levels return to normal on plant extract treatment.[52] By using *P. murex* L. these minerals can be reduce in the urine that can be reduced the struvite stone formation and it has been already formed that can also reduced to small size that passed through the urine. Administration of *P. murex* L. fruit extract reinstates the level of phosphate and reduced risk of stone formation.[52]

In the formation of urinary calculi, the glomerular filtration rate was reduced due to the hindrance to the flow of urine by the presence of stones in the urinary tract. For this reason, the waste materials, particularly nitrogenous substances such as urea, creatinine, blood urea nitrogen (BUN) and uric acid can be deposited in blood.[53] Te present study of treatment of EAEP showed to avoid the elevation of these markers in serum by improving glomerular filtration as it causes dieresis and significantly reduced the level of sodium, potassium, uric acid and creatinine in experimental EAEP treated groups. In the animal model, calcium oxalate urolithiasis reduction has been studied by using the roots of *Coleus barbatus* (L.) and *Trigonella foenum graecum* (L.) and it was revealed that the combination of this both extracts are most significant effect on the reduction of calcium oxalate stone when compared to the standard drug cystone.[54] By using ethylene – glycol induced calcium oxalate stone was treated by *Rotula aquatica* (L.) and *Orthosiphon grandiflorum* (L.).

In our *in-vivo* investigation, histological and urine analysis were shown significant changes can be visualised in the EAEP treated groups. Mean while, inhibitory effect of *O. grandiflorum* and their significant effect of urolithiasis by induction of ethylene glycol showed in animals through urinary analysis and histopathological studies.[55] Reduction of calcium oxalate deposition in the albino rats were studied in the plants *Macrotyloma uniflorum* (Fabaceae) seeds and *Peperomia tetraphylla*,[56] *Solanum virginianum*,[57] *Phyllanthus niruri* and *Aerva lanata*[58] through their histological studies which also revealed the proven report of modification in histology of kidney and urine. Overall, struvite stone is formed by the urinary super saturation in which it grows into larger particles turn into crystals. If it is retained in the urinary tract form the larger crystals then it can form into a struvite stone. In human, the enzyme urease converts urea into ammonia and carbon-dioxide reduced into carbonate which increases the urinary pH that forms crystals.[59,60] By using *P. murex* L. plant, it may inhibit the enzyme urease for the conversion of ammonia then inhibit the increase of urine pH. Through that crystal formation may be inhibited.

5. Conclusion

This is the foremost study to evaluate the effect of whole plant *P. murex* L. ethyl acetate extract on the diminution of struvite crystal *in-vitro* condition and animal model based on the presence of phyto-components in the plant. In the present work, ethyl acetate extract of *P. murex* L. inhibits *in-vitro* condition of growth of struvite crystals and the dissolution rate and also in the reduction of crystal deposition and renal damage proven by the animal model. It proved that the plant *P. murex* L. has an effective supplement source for urolithiasis study and treatment. As per the South Indian traditional healer's knowledge and the analysis of *P. murex* L. of our scientific findings on kidney stone reduction also proved and provided the supportive evidence to the intellectual knowledge on this plant. Hence, this plant may intake as a water supplement for the regular life style to prevent the kidney stone appearance. Future studies should include a more defined and controlled consumption studies as like food/water supplement and further development of an appropriate animal model to study the active fractions of *P. murex* L. extracts.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgement

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