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"STUDIES ON INHIBITION OF CALCIUM OXALATE CRYSTALLISATION BY USING PLANT EXTRACT AND SOIL MICROFLORA"

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ABSTRACT

Kidneys are the vital organs have indispensable function in body physiology, it maintains proper balance of water and mineral as well as filtration and excretion of waste products it also control blood pressure and produce certain hormones. Due to chemical imbalance in body the kidneys are deposited by hard minerals which results in to (kidney stone) Urolithiasis. Approximately 85% of stones are composed predominantly of calcium oxalate. Keeping the vision to investigate the novel strategies for the prevention and treatment of kidney stone disease. The *in-vitro* calcium oxalate inhibitory potential, of plant extracts viz; *Bryophyllum Pinnatum, Basil, Wheat Grass,* and *Lemon* and of cell free extract of soil bacteria viz; *Streptococcus, Enterococcus, Pseudomonas* was analyzed separately; The inhibition of nucleation, aggregation and growth was investigated spectrophotometrically. The study revealed that both plant and cell free bacterial extract have the possible inhibitory effect on calcium oxalate for its crystal nucleation, aggregation and growth. Therefore study enlightens the beneficial use of plant & soil bacterial extracts for treatment of urolithiasis.

KEYWORDS: Urolithiasis, Calcium Oxalate Crystallization, Nucleation, Aggregation, Growth and Medicinal Plants and Soil Bacterial Extracts.

INTRODUCTION

The changing life style made a remarkable shift in the food habits of people all over the world because of increased interest on junk foods and soft drinks. In any industrialized part of the world it is estimated that nearly 10 % of the population is affected by urinary tract stone disease.[1] Urolithiasis is one among the oldest and widely reported diseases known to mankind.[2-3] Urolithiasis or renal lithiasis is also known as urinary calculi, urinary stones, kidney stones, renal stones or renal calculi.[4] It refers to the growth of hard mineral calcification formed in the urinary system. Based on their composition, five common types of kidney stones are categorized as calcium oxalate, calcium phosphate, and uric acid, struvite, and cysteine stones. Approximately 85 % of stones are composed predominantly of calcium compound as calcium oxalate[5] and hence calcium oxalate (CaOx) is preferred for the study .Effective kidney stone prevention can be done by number of treatment methods but none of the treatment method is without side effects and not provides satisfactory treatment options for urolithiasis.[6-10] Thus, the focus should be on top of the development of novel strategies for the prevention and treatment of kidney stone disease. Microorganism as well as Herbal medicines have optimistic alternative to overpass the gap in this regard. Treatment with oxalate degrading bacteria or probiotics could be the innovative choice.[11] Previous studies have suggested a correlation between increased of urinary oxalate levels in absence of *oxalobacter formigenes* colonization. As an advance the oxalate degrading bacteria have been isolated from the human intestine tract, including *Eubacterium Lentum*[12], *Enterococcus faecalis*[13], and *providenciarettgeri.*[14] Recently, probiotic bacteria belonging to *Bifidobacterium* and lactobacillus have been studied for their potential capability to degrade oxalate .The search for new antilithiatic drugs based on ethno botanical approaches therefore assumes greater importance as herbal drugs are not only cost effective but also confer minimal side effects.[15] The herbal products today symbolize safety and have been valued for their medicinal, flavoring, and aromatic qualities for centuries.[16]

Hence, In order to authenticate traditional usage of plant and microbial extract for the treatment of kidney stone, the evaluation of calcium oxalate inhibition potential of revived plant extracts and certain soil micro flora has planned for the at hand study.

MATERIALS AND METHOD

The aim of the present study was to explore the antilithiatic prospective of some medicinal plant Viz; *Bryophyllum Pinnatum, Basil, Wheat Grass, Lemon* as

well as of certain soil microorganisms Viz*; Streptococcus, Enterococcus, Pseudomonas in vitro*.

Preparation of plant extracts

Fresh leaf of all the above cited plants were collected and cleaned to remove adhering soil and dust particles, washed well in running tap water. Further the collected leaves were cut into thin slice and grinded in grinder the grounded preparation was subjected for centrifugation at 2500 rpm for 5 minute. After centrifugation, supernatant were used as crude plant extract for further assays.

Preparation of cell free extract of soil isolates

The Rhizospheric Soil samples from the medicinal plant Viz; *Bryophyllum Pinnatum*, *Basil*, *Wheat Gras* and *Lemon* were collected in sterile poly bag and transported to parent laboratory. Soil sample were enriched by inoculating 1 gm of soil to 100 ml of in TSB at 30^{o}C for 48hours.The enriched samples were further, spreaded on plates of different selective media viz; Thiosulphate agar for *Streptococcus species*, on cetramide agar for *Pseudomonas species*, and on hekton enteric agar for *Enterococcus species* and further incubated at 45-55^{o}C, at 35^{o}C and at 37 ^{o}C respectively. Followed by incubation the developed growth was examined for its colony and morphological characterization. All the isolated colonies were further re-enriched at respective temperatures for 48 hours in nutrient broth. After incubation centrifugation was carried out at 2500 rpm for 5 minute. After centrifugation, supernatants were separated and used as crude cell free extract for further assays.

Evaluation of antilithiatic potential

The study was augmented over three different assays viz: Nucleation, Aggregation and Growth assay respectively using calcium oxalate as target compound.

Nucleation assay

The stone formation starts from the nuclei, means the process of new crystal formation. The percentage inhibition of nucleation of calcium oxalate crystals by extracts was evaluated by the modified method of Atmani and Khan.[17] Total 3mMol/L and 0.5mMol/L of calcium chloride and sodium oxalate solutions were prepared in a buffer containing NaCl 0.15mMol/L and Tris 0.5mMol/L at pH 6.5. 100 µl of the plant leaf and cell free extract of bacterial isolates were pipetted out separately and were mixed with 950 µl of calcium chloride solution. Crystallization was initiated by the addition of 950 µl of sodium oxalate solution.100 µl of distilled water without extract was maintained as control. The tubes were incubated at 37˚C followed by the incubation the optical densities of the solutions were monitored at 620 nm. The rate of nucleation was estimated by comparing the induction time for calcium oxalate crystal formation in the presence and absence of the extracts. Percentage inhibition of nucleation was calculated using formula: % Inhibition of nucleation = [(C-S) /C] × 100 Where, C is the turbidity without extract. S is the turbidity with extract.

Aggregation assay

The aggregation inhibition in presence and absence of the plant leaf and cell free extract of bacterial isolates were determined by the method of Hess *et* al.[18] 50 mMol/L of calcium chloride and sodium oxalate solution was mixed and equilibrated at 60˚C in a water bath for 1 hour and then cooled to 37˚C overnight. The harvested crystals were used for aggregation studies. The harvested crystals were further added to 0.8 mg/ml buffered with Tris 0.5 mMol/L and NaCl 0.15 mMol/L at pH 6.5. The buffered crystal was incubated at 37˚C and the aggregation study was carried out. The aggregation study was done separately in absence and presence of the plant leaf and cell free extract of bacterial isolates. The percentage aggregation inhibition rate (Ir) was calculated by comparing the turbidity in the presence of the extracts with that obtained in the control using the Formula: Ir = (1-turbidity sample/turbidity control) x 100.

Growth assay

Briefly, 20 ml each of 4Mm calcium chloride and 4Mm of sodium oxalate were added to a 30ml of solution, containing NaCl (90mM) buffered with tris HCl (10mM) pH 7.2.To this 600µl of calcium oxalate monohydrate (COM) crystal slurry as well as to this 1.5 mg/ml acetate buffer was added. Consumption of oxalate begins immediately after COM slurry addition and was monitored for 30 minutes for disappearance of absorbance at 214nm.1 ml of plant leaf and cell free extract of bacterial isolates was separately added into reaction mixture. The depletion of free oxalate ions will decrease if extract inhibits calcium oxalate crystal growth.[19] Rate of reduction of free oxalate was calculated using the baseline value and the value with or without extract after 30 min of incubation. The percentage inhibitory activity was calculated by Formula: Percentage inhibitory activity = [(C-S)/C] x 100.Where, C is the rate reduction of free oxalate without extract. S is the rate reduction of free oxalate with extract.

RESULT AND DISCUSSION

Total three isolates from the Rhizospheric Soil samples from the medicinal plant Viz; *Bryophyllum Pinnatum*, *Basil*, *Wheat Grass*, and *Lemon* were isolated. The samples were enriched and further, cultured on plates of different selective media .The developed growth was examine for its colony and morphological and Biochemical characterization. It was observed that the screened characters of the three isolates, indicates the possible species as *Streptococcus*, *Pseudomonas* and *Enterococcus* respectively.

Nucleation is an initial step in kidney stone formation. Nucleation is the establishment of the smallest unit of crystal formation in a solution step in renal stone formation. CaOx nucleation was monitored in presence and absence of plant extracts as well as in presence of cell free bacterial extract. From the results on *in-vitro* study it was observed that with all the plant extract

investigated, the Nucleation was found to be inhibited and the crystal formation was prevented as compared to control without plant extract (Fig: 1) .The maximum inhibition of nucleation (88.11) % was recorded due to *Wheat Grass* extract followed by *Basil* extract (77.97) %, *Lemon* extract (14.49) % respectively. Whereas the *Bryophyllum Pinnatum* extract showed minimum (13.04) % inhibition of nucleation over all other plant extracts .In case of control without plant extract the inhibition of nucleation was only(0.69) percent. From the results it was accomplished that the *Wheat Grass* extract can be the appropriate plant Source for the managing of kidney stone especially at the phase of nucleation. The results on the present study are comparable with the experimental findings of[20] they reported the therapeutic potential of plant extract for the kidney stone. However their studies are on the *Beet* extract.

Similarly, it was observed that with the entire cell free bacterial extract under study , the nucleation was found to be inhibited and the crystal formation was prevented as compared to control without cell free bacterial extract (Fig:2).The maximum inhibition of nucleation (78.26) % was recorded due to *Streptococcus* and *Enterococcus*. Whereas the *Pseudomonas* showed minimum (73.91) % inhibition of nucleation compared with other cell free bacterial extract. In case of control without cell free bacterial extract the inhibition of nucleation was only (0.69) percent. From the results it was concluded that the cell free bacterial extract especially of *Streptococcus* and *Enterococcus* can be the suitable soil isolates for the preventing of kidney stone especially at the phase of nucleation. The results on the present study are comparable with the experimental findings of[21] they reported the biodegradation of calcium oxalate by newly isolated bacterial culture however there method of the experimentation was different.

The second important step of stone formation is aggregation that constitutes the most effective mechanism to increase the size of particles, composition and structure of urinary stone. Stone crystals bind to one another through a process known as aggregation or agglomeration. The aggregation was monitored in presence and absence of plant extracts as well as in presence of cell free bacterial extract. From the results on *in-vitro* study it was observed that with all the plant extract analyzed the aggregation was found to be inhibited and the crystal formation was prevented as compared to control without plant extract(Fig:3). The maximum inhibition of aggregation (21.60) % was recorded due to *Lemon* extract followed by *Bryophyllum Pinnatum* extract (20.57), *Basil* extract (18.88), respectively. Whereas the *Wheat Grass* extract showed minimum (18.66) % inhibition of aggregation over all other plant extracts. In case of control without plant extract the inhibition of aggregation was only 0.045 percent. From the results it was accomplished that the lemon extract can be the appropriate plant for the managing of kidney stone especially at the phase of aggregation. The results on the present study are comparable with the experimental findings of Nirmaladevi et al[22] they reported the antilithiatic potential of plant extract for the kidney stone. However their studies are on the *Hibiscus rosa-sinensis* extract.

Similarly from the *in-vitro* study it was observed that with all the cell free bacterial extract analyzed the aggregation was found to be inhibited and the crystal formation was prevented over the control without cell free bacterial extract(Fig:4). The maximum inhibition of aggregation (21.00) % was recorded due to *Streptococcus* followed by *pseudomonas* showed (20.13) % of inhibition of aggregation. Whereas the *Enterococcus* showed minimum (15.5)% inhibition of aggregation compared with all other cell free bacterial extract. In case of control without cell free bacterial extract the inhibition of aggregation was only (0.045) percent. From the results it was concluded that the cell free bacterial extract especially of *Streptococcus* and *pseudomonas* can be the suitable soil bacteria for the managing of kidney stone especially at the phase of aggregation. The results on the present study are comparable with the experimental findings of Rama Devi et al.[23] They reported Gut-Inhabiting Bacterium *Oxalobacter formigenes* role in Calcium Oxalate Urolithiasis. However there method of the experimentation was different.

The, crystal growth is critical step in the urinary stone formation which due to agglomeration of particles. Newly formed crystals may combine or grow to form a small, hard mass called stones. The Crystal growth was monitored in presence and absence of plant extracts as well as in presence of cell free bacterial extract. From the results on *in-vitro* study it was observed that with plant extract analyzed the crystal growth was found to be inhibited and the crystal formation was prevented as compared to control without plant extract(Fig:5). The inhibition of crystal growth (0.73) % was recorded due to *Wheat Grass* extract. Whereas the *Lemon* extracts, *Bryophyllum Pinnatum* extract, *Basil* extract showed no inhibition of crystal growth over extracts. In case of control without plant extract the inhibition of crystal growth was only 0.136 %. From the results it was accomplished that the *Wheat Grass* extract can be the suitable plant for the managing of kidney stone especially at the phase of crystal growth. The results on the present study are comparable with the experimental findings of Rathod N *et al*.,[24] they reported the anti-urolithiasis potential of herbal drugs for the kidney stone.

From the results on in-vitro study it was observed that with all the cell free bacterial extract analyzed the crystal growth was found to be not inhibited and the crystal formation was not prevented as compared to control without cell free bacterial extract(Fig:6). *Streptococcus*, *Enterococcus* and *Pseudomonas* showed no inhibition of crystal growth. In case of control without cell free bacterial extract the inhibition of crystal growth was

(0.136) percent. From the results it was accomplished that the cell free bacterial extract of *Streptococcus*, *Pseudomonas* and *Enterococcus* cannot be the suitable microorganism for the preventing of kidney stone only at the phase of crystal growth.

Table no.1: effect of plant extract and cell free bacterial extract on nucleation, aggregation and growth phase.

SR.NO.	Plant extracts	Nucleation inhibition (%)	Aggregation inhibition (%)	Growth inhibition (%)	Mean (%) Anti-Lithiatic Activity
1.	Wheat grass extract	88.11	18.66	0.73	35.83
2.	Basil extract	77.97	18.88	No Inhibition	48.42
3.	Lemon extract	14.49	21.60	No Inhibition	18.04
4.	Bryophyllum Pinnatum extract	13.04	20.57	No Inhibition	16.80
5.	Control	0.69	0.045	0.136	0.29
SR.NO.	**Cell free bacterial extracts**	**Nucleation inhibition (%)**	**Aggregation inhibition (%)**	**Growth inhibition (%)**	**Mean (%) Anti-Lithiatic Activity**
1.	*Streptococcus species*	78.26	21.00	No Inhibition	49.63
2.	*Pseudomonas species*	73.91	20.13	No Inhibition	47.02
3	*Enterococcus species*	78.26	15.5	No Inhibition	46.88
4.	Control	0.69	0.045	0.136	0.29

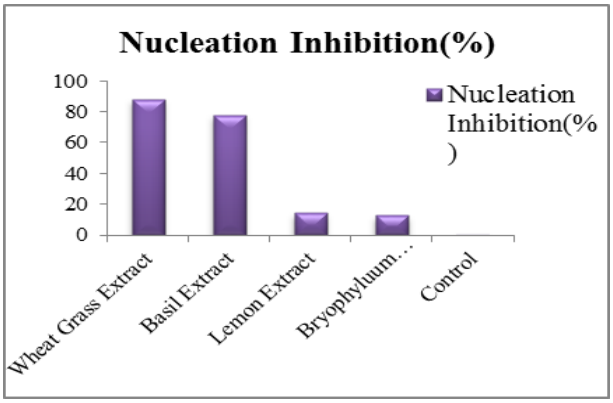

Figure 1: Effect of different plant extracts on (%) Nucleation inhibition

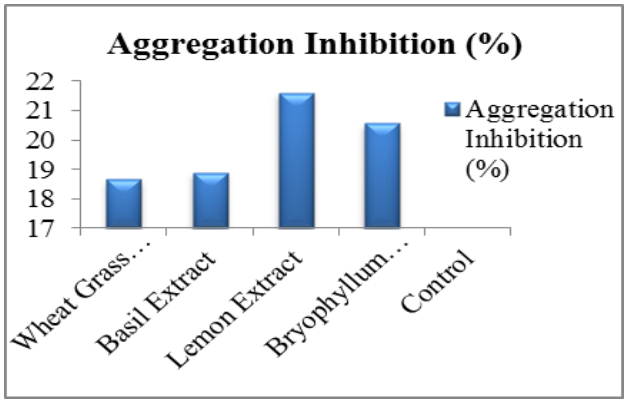

Figure3: Effect of different plant extracts on (%) Aggregation inhibition

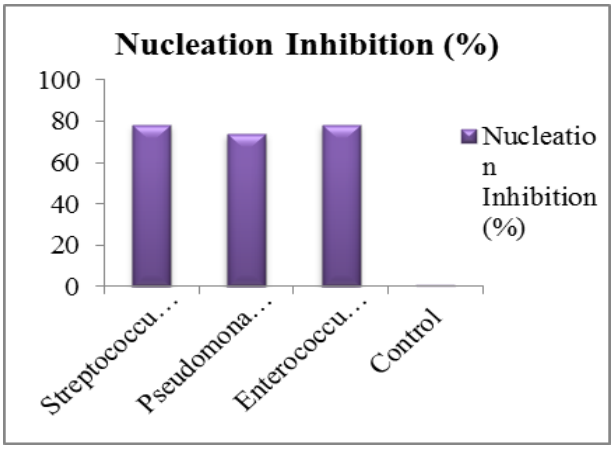

Figure 2: Effect of different cell free bacterial extracts on (%) Nucleation inhibition

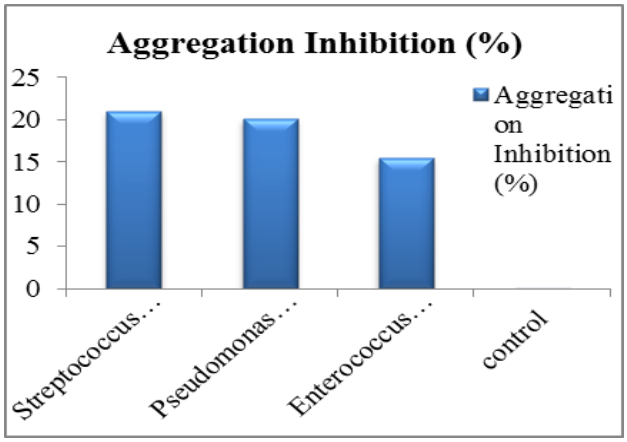

Figure 4: Effect of different cell free bacterial extracts on (%) Aggregation inhibition

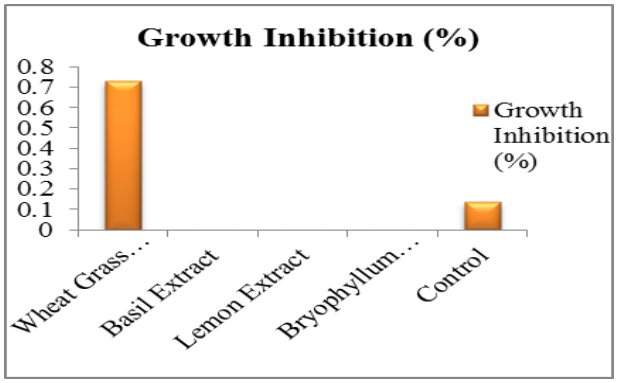

Figure 5: Effect of different plant extracts on (%) Growth inhibition

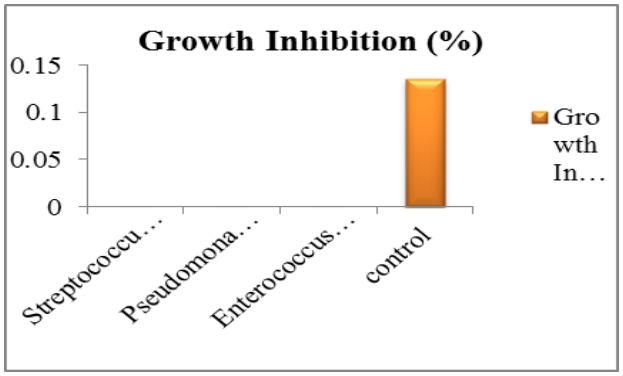

Figure 6: Effect of different cell free bacterial extracts on (%) Growth inhibition

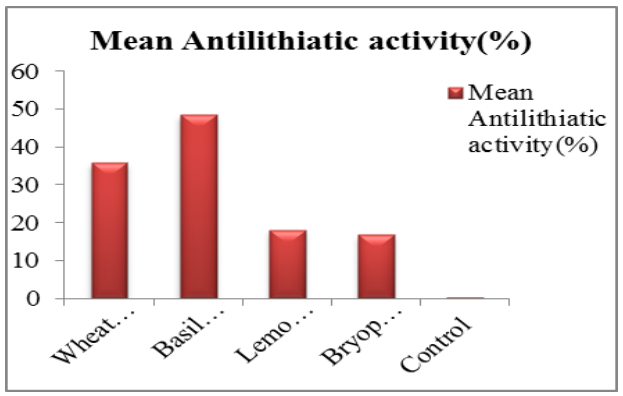

Figure 7: Effect of different plant extracts on (%) Mean Anti-Lithiatic Activity

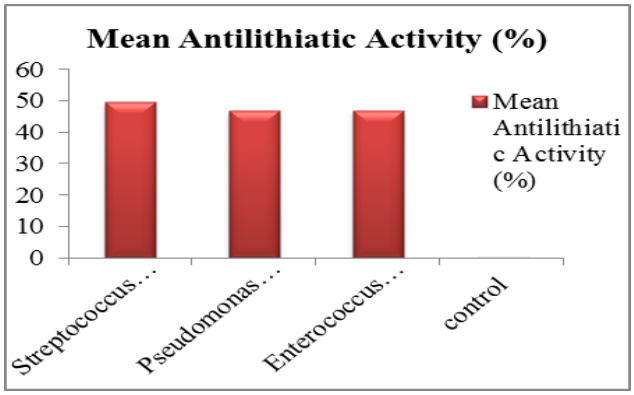

Figure 8: Effect of different cell free bacterial extracts on (%) Mean Anti-Lithiatic Activity

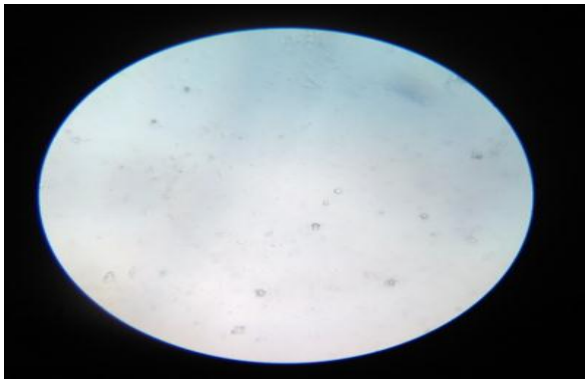

Plate No.1: a)

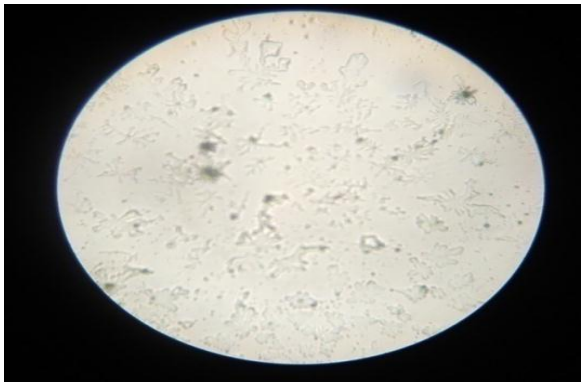

Plate No.1: b)

(a & b) showing microscopic view of calcium oxalate crystal without extract and microscopic view of calcium oxalate crystal with extract respectively.

The steps involved in stone formation include nucleation, growth and aggregation. Hence, taking into consideration various steps in stone formation with focus on the overall consequence of stone development, the antilithiasis activity of various plant and cell free bacterial extract has been calculated by fetching up the mean effect of extract on nucleation, growth and aggregation(Fig:7&8). From the results it was observed that the maximum mean i.e. (49.63) percent antilithiasis activity was due to bacterial cell free extract of *Streptococcus* followed by *Basil* extract (48.42) mean %, *Pseudomonas* extract(47.02)mean %, *Enterococcus* extract(46.88)mean %, *Wheat Grass* extract(35.83) mean % and *Lemon* extract(18.04) mean % respectively. Whereas the *Bryophyllum Pinnatum* showed minimum (i.e.16.80) mean % antilithiasis activity. The results on the present in vitro assays performed in the study revealed that the cell free extract of *Streptococcus species and* plant extract of *Wheat Grass* a exhibited a superior inhibitory effect against crystal nucleation, aggregation, growth. It is clearly evident that the compounds present in these extracts might be responsible for its preventive action against kidney stone formation therefore the components present in the extract can very well be used for medicinal preparations for the treatment of lithiasis after authentication and validation study.

CONCLUSION

The study concludes that the plant and cell free extracts of soil bacterial isolates have inhibitory effect on calcium

oxalate for crystal nucleation, aggregation and crystal growth thus these extracts may be valuable resource for treatment of urolithiasis. However the, diversity of plant and their rhizo micro flora should be investigated on *in vivo* models on the line of present study.

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