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Article

Anti urolithiatic activity of *Cyperus rotundus* tubers: *In silico*, *In vitro* and *In vivo* approaches

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The present research evaluated the anti urolithic potential of *Cyperus rotundus* tubers extract using *in silico, in vitro* and *in vivo* techniques. *In silico*study was performed of *Cyperus rotundus* constituents and pathological protein oxalate oxidase (PDB Id: 2ETE). *In vitro*study, nucleation and aggregation assay involved for assessment of ethanol extract of *Cyperus rotundus* tuber (50–3000 µg/ml).*In vivo* studies involved that the *Cyperus rotundus*ethanolic extract (100, 200 and 400 mg/kg B.wt.) wastreatedonsodium oxalate induced urolithiatic rats for seven days,evaluated kidney function by urine and serum biochemical analysis and statistical analysis performed usingGraphPad prism5 software.*In silico* results showedthat *Cyperus rotundus* constituents,Humulene epoxide, 4-Oxo-alpha-ylangene, Cubebol were exhibited better binding energyonoxalate oxidase.Ethanolic extract of *Cyperus rotundus*tuber was exhibited nucleation, aggregation of calcium oxalate monohydrate crystals inhibition in dosedependent manner. Sodium oxalate treatment was triggered biochemical changesin the urine that have been substantially prevented by the ethanolic extract of *Cyperus rotundus* tuber. The current findings *Cyperus rotundus* anti urolithic activity due to antioxidant essential oils. The molecular docking results could be used to optimize lead and develop the appropriate urolithiasis treatment.

Keywords: Urolithiasis. Nucleation and aggregation.Oxalate oxidase. Sodium oxalate. Ethanolic extract of *Cyperus rotundus* tubers (EECR).

INTRODUCTION

Urolithiasis is an growing health problem in industrialized countries and often linked to habits such as hypertension, diabetes, obesity and metabolic syndrome(Rosa *et al.,* 2013). Unexpectedly,12% of the world's population is suddenly impacted by the urinary system's stone illness (Basavaraj *et al.,* 2007). Stone development includes multiple physico chemical occurrences, including supersaturation, nucleation, growth, aggregation and retention within the kidneys (Basavaraj *et al.,*2007; Worcester, Coe, 2008). Thus, extracorporeal shock-wave lithotripsy, surgical procedure and local calculus disruption using high-power laser, endourological procedures such as ureteroscopy or percutaneous extraction processes remain only non-invasive stone therapy technique (Chaussy *et al.,* 1982; Prasad, Sujatha, Bharathi, 2007). However, these procedures are highly costly and with these procedures recurrence is quite common, undesirable side effects such as tubular necrosis hypertension, hemorrhage, subsequent fibrosis of the kidney leading to cell injury (Terlecki, Triest, 2007; Xue *et al.,* 2009). The therapy of kidney stone disease in modern medicine is costly and not accessible by all. In modern medicine, there are actually no adequate drugs to dissolve the kidney stone. Herbal medicines and their components have been extensively investigated in kidney stones treatment (Lai, Lin, Huang, 2018). Data from *in vitro*, *in vivo* and clinical studies showed that phytotherapy agents could be fully used as an alternative or complementary therapy for urolithiasis management. (Butterweck, Khan, 2009). Plants and herbal preparations are therefore used since ancient times for the treatment of kidney stones.

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Cyperus rotundus L. (Cyperaceae) is a perennial glabrous herb commonly referred to as Nut Sedge. It is a highly invasive weed, distributed widely throughout the world in tropical, subtropical and temperate regions(Parsons, Cuthbertson, 2001). Practitioners of ayurvedic and herbal medicine have described *Cyperus rotundus* tubers for the treatment of uterine disorders, fever, delayed menstruation and dysmenorrhea, removal of obstructions and stomach and soft plasters. (Mohsin *et al.,* 1989; Peerzada *et al.,*2015). *Cyperus rotundus* tubers have also been used in traditional Chinese medicine to treat dysentery and female diseases as an antidiarrheal, antidepressant, analgesic, anti inflammatory and antiemetic remedy(Oh *et al.,* 2015). It has antioxidant activity (Yazdanparast, Ardestani, 2007), protective effect on hypoxia injury (Jebasingh *et al.,* 2014). Antioxidant activity (Yazdanparast, Ardestani, 2007) has a protective effect on injury to hypoxia (Jebasingh *et al*., 2014). Plant extracts and their essential oils were the main source of potentially biologically important natural products to be used as alternative remedies for trees.This research was performed to assess the anti urolithic activity of *Cyperus rotundus* tuber extract against sodium oxalate induced urolithiasis in rats and determine the binding energy to the urolithiatic pathological protein.

MATERIAL AND METHODS

Molecular docking studies

Docking simulations were performed as per the reported procedure (Trott, Olson, 2010; Kumar *et al.,* 2013; Kumar *et al.,* 2015). Docking studies of the constituents of *Cyperus rotundus* tubers which are present high yield (Aghassi,Naeemy,Feizbakhsh,2013; Al-Massarani *et al.,* 2016) were carried out on protein 2 ETEof Oxalate oxidasewas downloaded from pdb (www.rcsb.org/pdb)which reportedly participate in kidney stone formation (Dahiya, Pundir, 2013), using docking programs AutoDock Vna and AutoDock 1.5.6. The results were visualized using PyMol and Discovery studio visualizer (Discovery studio visualizer ver. 2.5). Grid parameters center X:-35.012, Y: -28.570, Z:-70.6834; Dimensions (Angstrom): X: 75.6389, Y:65:9654, Z: 55.9347 was chosen in PyRx tool and Dimensions: X: 126, Y:126, Z: 126, spacing: 0.492, center X:-32.382 Y: -30.604; Z:-72.107 was chosen in Autodoc 1.5.6 tool.Binding energy was calculated as the sum of the intermolecular energy and the torsional free-energy penalty (Huey,Morris, Forli, 2012).

Collection of *Cyperus rotundus*tubers

Cyperus rotundus tubers was collected from the garden of Creative Educational Society's College of Pharmacy, Kurnool, Andhra Pradesh, India and authenticated by Prof. K. Madhava Chetty, Assistant Professor, Botany Department, University of Sri Venkateswara, Tirupathi.(Voucher Number: 2056).

Solvent extraction

Cyperus rotundus tuber powder was extracted by ethanol using sohxlet apparatus then concentrated with rotary vacuum evaporator and yield was 6%. Ethanol extract of *cyperus rotundus* tubers (EECR) was used for antiurolithiatic activity.

In vitro methods

Anti urolithiatic activity of EECR was estimated by Nucleation and Aggregation assays (Saha,Verma, 2015).

Nucleation Assay

Solution of calcium chloride and sodium oxalate were prepared at a final concentration of 3 mmol/L and 0.5 mmol/L respectively, prepared in Tris 0.05mol/L and NaCl 0.15 mol/L at p^H 6.5.950 µL of calcium chloride solution was mixed with 100 µL of differentextract concentrations (50-3000 **µg/ml**). A blank reading was taken and crystallization was checked by adding 950 µL of sodium oxalate solution. The OD of the solution was monitored after a period of 30 minutes at 620 nm using UV/Visible spectrophotometer. The temperature was maintained at 37^0C.The rate of nucleation was estimated in the presence of the different concentration of EECR with that of the control.

Aggregation assays

Calcium oxalate crystals aggregation was determined by a spectrophotometric assay. The calcium oxalate monohydrate (COM) crystals were prepared by mixing of calcium chloride and sodium oxalate of 50 mM solutions each. The solutions were then cooled to 37^{0}C and then evaporated. The COM crystals were dissolved with 0.5ml of 0.05mM Tris buffer and 0.5ml of 0.15mM NaCl solution at p^{H} 6.5 to a final concentration of 1 mg/ml. Absorbance at 620 nm was recorded. The rate of aggregation was estimated by comparing the slope of turbidity in the presence of the extract against control.

% inhibition = (1 - Turbidity of the sample ÷ Turbidity of the control) × 100

Preparation of animals

The animals are randomly selected, marked to permit individual identification, and kept in their cages for at least five days prior to dosing to allow for acclimatization to the laboratory conditions.The study protocol was approved by the Institutional Animal Ethics Committee (DEC/IAEC/CESCOP/2017-016).

Acute toxicity

Acute toxicity of EECR was performed as per OECD guidelines 423 (limit test). Six Wistar albino rats (either sex) (three animals in each step) were randomly selected. The rats were kept fasting for overnight providing only water. The extract was administered orally at one dose level of 2000 mg/kg B.wt of the rats. In further rats were observed continuously for the first 4 hr and then periodically up to 24 hr for toxic symptoms and mortality.

Evaluate anti urolithiatic activity of *Cyperus rotundus*tubersby sodium oxalate induced urolithiasis (Pawar, Vyawahare, 2017).

Sodium oxalate induced hyperoxaluria method was used to judge the antiurolithiatic activity in Wistar albino rats. Rats were separated into six groups, group containing six animals.

Group I Normal Control
Group II Urolithiatic control [Sodium oxalate (70mg/kg, B.wt .i.p. administration for 7 days)]
Group III Sodium oxalate - Cystone (500mg/kg, B.wt.p.o 8^{th} day to 14^{th} day)
Group IV Sodium oxalate- EECR(100mg/kg, B.wt.p.o. 8^{th} day to 14^{th} day)
Group VSodium oxalate -EECR(200mg/kg, B.wt.p.o. 8^{th} day to 14^{th} day)
Group VI Sodium oxalate -EECR (400mg/kg, B.wt.p.o. 8^{th} day to 14^{th} day)

Assessment of Antiurolithiatic activity

On the 14th day, blood was collected through retro orbital plexus under slight anesthetic conditions. Serum was separated by centrifugation at 10,000×g for 10 min and analyzed for creatinine, uric acid and blood urea nitrogen (BUN).On the 14th day individual rats were kept in separate metabolic cages. Urine of 24 hr sample was collected. Provide the water for rats during the urine collection epoch and analyzed for sodium, chloride, BUN, creatinine, uric acid.

Data analysis

All values are expressed as mean ±S.E.M for six rats in each group. Comparisons made between ***$p<0.001$,**$p<0.01$,*$p<0.05$; Normal Vs Disease control, Disease control Vs Treatment:One-way ANOVA followed by Dunnett's -t test using the software graph pad prism 5 (Motulsky, 2003).

RESULTS

Docking study

Current study involvesvirtual screening of about twelveconstitutes of *Cyperus rotundus* using Pub chem. database against the Oxalate oxidase (2 ETE) extracted from protein data bank. The data obtained from Auto doc

vina studies showed that Cubebol, Humulene epoxide and 4-Oxo-alpha-ylangene were exhibited significant binding energy on 2 ETE. The 2D diagrams of highest ranking docked conformations of constituents Cubebol, Humulene epoxide and 4-Oxo-alpha-ylangene in the binding site of enzyme are depicted (Table I). Humulene epoxide (Pub Chem ID5463721) was highest scoring constituent of *Cyperus rotundus*, whendocked into Oxalate oxidase (2 ETE) showed Conventional H Bond interaction with Ser: 40 and interaction distance was 1.86, 2.78 Aº, Alkyl interaction with Pro: 65, Lys: 41and interaction distance was 5.16, 3.83 Aº, Pi Alkyl interaction with Trp: 64 and interaction distance was 4.02 Aº, with Binding energy -6.3 K Cal/mol. 4 Oxo alpha Ylagene (Pub Chem ID 101358349) exhibited that Conventional H Bond interaction with Gly 66, interaction distance was 1.93 Aº, Alkyl interaction with Pro: 65, Lys: 41 and interaction distance was 4.02, 4.07 Aºwith Binding energy -6.2 K Cal/mol. Compound Cubebol (Pub Cem ID 11276107) exhibited Pi Sigma, Pi with Phe:138, interaction distance was 3.80, 3.85, 4.62 Aº, Alkyl interactions with Pro: 87 with interaction distance 3.91, 4.05, 4.88; Leu:109 and interaction distance was 5.16 Aº, Leu: 118 interaction distance was 5.2 Aº with Binding energy -6.1 K Cal/mol (Table II; Figure: 1a,b,c).

TABLE I - Receptor ligand binding energy data obtained from Auto Dock Vina

S.No	Name of the compound	Oxalate oxidase
1	Humulene epoxide	-6.3
2	Caryophylene oxide	-5.5
3	Cubebol	-6.1
4	Cyperone	-6
5	Myrtenal	-4.6
6	Transcarveol	-5.4
7	Transverbenol	-5
8	Transpinocarveol	-4.9
9	Verbinone	-4.7
10	4- Oxo- alpha ylagene	-6.2
11	P Cymen	-5.3
12	Myrtenol	-4.8

TABLE II - Receptor ligand binding energy data obtained from Auto Dock 1.5.6

Compound Name	Inhibitory Constant (mM)	Binding Energy (Kcal/)	Interacting Residues	Distance (Å)	Type of binding interaction
Cubebol (Pub Chem. ID 11276107)	2.2	-6.1	PHE 138 PRO 87 LEU 109 LEU 118	3.80, 3.85, 4.62, 4.58,4.65 3.91, 4.05, 4.88 5.16 5.2	Pi Sigma, Pi Alkyl. Alkyl Alkyl Alkyl
Humulene epoxide (Pub Chem. ID 5463721)	1.25	-6.3	PHE 38 SER 40 PRO 65 TRP 64 LYS 41	3.49 1.86, 2.78 5.16, 3.83 4.02 4.88	Carban Conventional H Bond Alkyl Pi Alkyl Alkyl
4 -Oxo -alpha Ylagene (Pub Chem. ID 101358349)	0.935	-6.2	GLY 66 PRO 65 LYS 41 TRP 64	1.93 4.02 4.07 3.07	Conventional H Bond Alkyl Alkyl Carbon

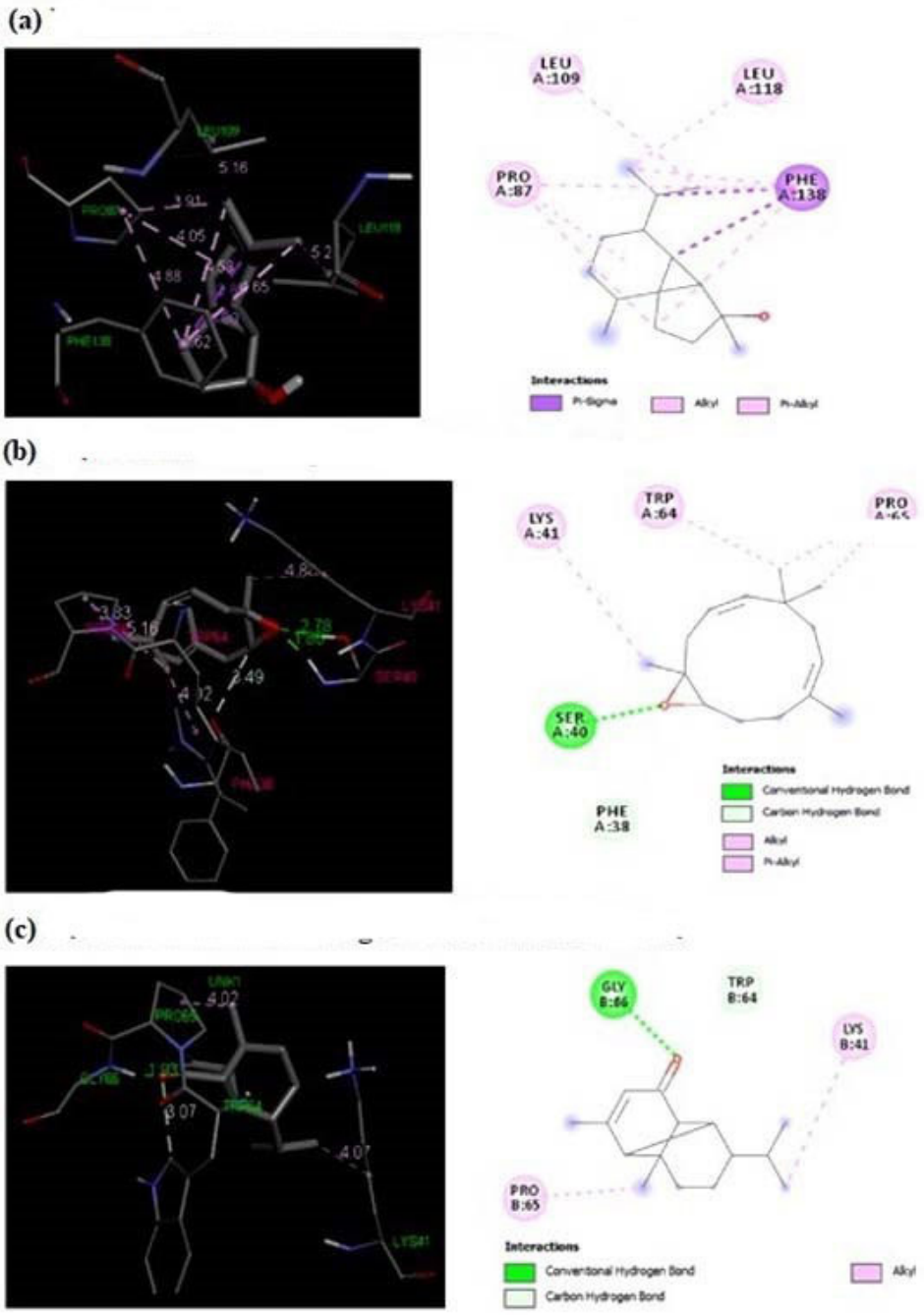

FIGURE 1 – Interacting distance and type of binding interaction a) Between Cubebol and oxalate oxidase b) Between Humulene epoxide and oxalate oxidase c) Between 4 Oxo alpha Ylagene and oxalate oxidase.

Effect of EECR on CaOx crystal nucleation

The results as shown in (Figure2) revealed that the turbidity of the solution in the presence of the EECR increased in contrast to the control, indicating that oxalate crystallization was disturbed in the presence of the extract. The data represents that the inhibition of crystal formation was directly proportional to the increase in concentration of the EECR, with maximum activity observed at 1600µg/ml, beyond which, at 3200 µg/ml there was no further inhibition observed.

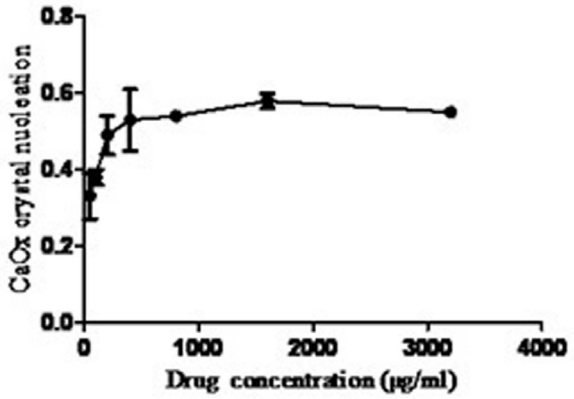

FIGURE 2 - Effect of EECR on CaOx crystal nucleation.

Aggregation assay results showed a similar trend to that of the nucleation assay. Crystals treated with the EECR were less aggregated, while the rate of inhibition elevated with increase in concentration of the extracts. The results were supported by the spectroscopic analysis as the absorbance was found to be increased in the extract treated groups when compared to the control due to disaggregation. At the dose of 3200 µg/ml of EECR was exhibited 82.35% aggregation inhibition rate and it was showed dose dependent crystal aggregation inhibition (Figure 3).

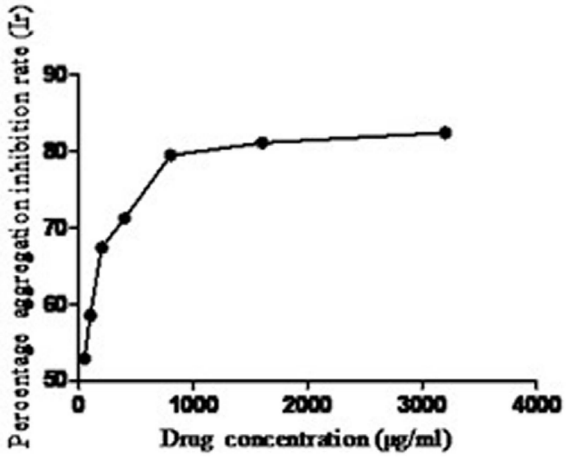

FIGURE 3 - Effect of EECR on CaOx crystal aggregation.

Effect of EECRon serum parameters in Sodium oxalate induced urolithiatic rats

Sodium oxalate treated rats showed significant increase in serum levels of BUN (128.6±18.85: $p<0.001$***), Creatinine (3.97±0.28; $p<0.001$***) Uric acid (6.35±0.31: $p<0.001$***) when compared with normal control rats BUN (28.62±398) Creatinine (0.48±.16) Uric acid (2.16±0.32). While treated with Cystone (500 mg/kg, B.wt.) serum BUN (70.19±4.06; $p<0.001$***), Creatinine (1.60±0.32; $p<0.001$***) and Uric acid (3.04±0.35; $p<0.001$***) were significantly reduced when compared to sodium oxalate induced urolithiasis rats. However EECR (400 mg/kg, B. wt.) treated rats were exhibited significant reduced BUN (55.21±4.23: $p<0.001$***) Creatinine (078±0.22: $p<0.001$***) Uric acid 2.68±0.36: $p<0.001$) levels, when compare to sodium oxalate induced urolithiasis rats (Figure 4).

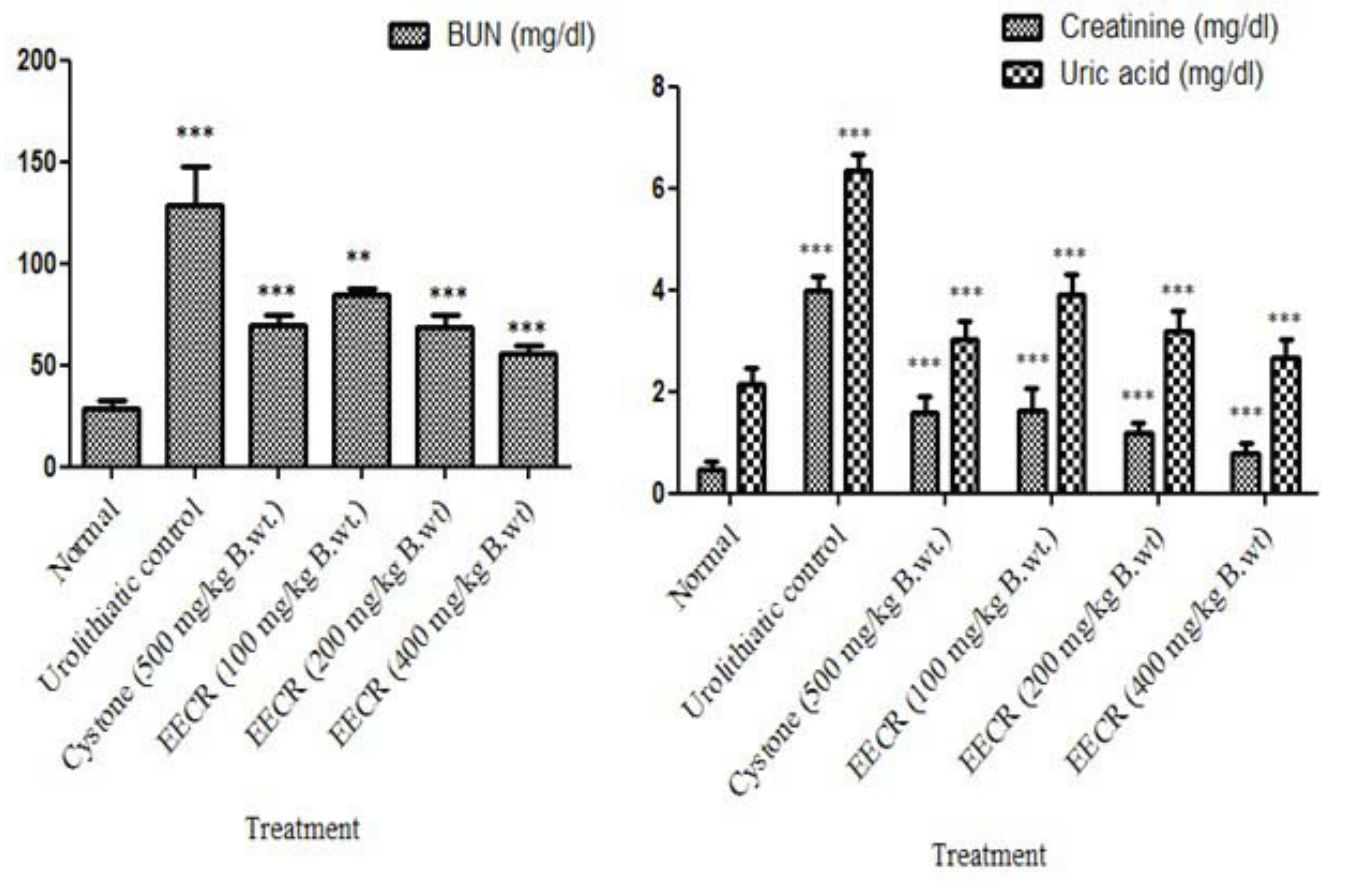

FIGURE 4 - Effect of EECR on serum BUN, creatinine, uric acid in urolithiasis rats.

Effect of EECR on urine parameters in Sodium oxalate induced urolithiatic rats

Sodium oxalate treated rats showed significant increase urinary BUN (69.46±2.87: p<0.001***), creatinine (7.86±0.29: p<0.001***), uric acid (109.4±4.48: p<0.001***) sodium (39.15±2.81: p<0.001***), chloride (41.20±3.34: p<0.001***) were reduced compared to normal rats. While treatment of EECR(400 mg/kg, B.wt) raturine BUN (29.15±2.05: p<0.001***), creatinine(2.79±0.31: p<0.001***), uric acid (63.42±2.34: p<0.001***) were significantly decreased, urinary sodium (96.62±3.55: p<0.001***), chloride (73.23±2.60: p<0.001***) levels were decreased compared to sodium oxalate feeded rats. Activity results were compared with standard drug Cystone (500 mg/kg, bd. wt, p.o) exhibited significant reduces the serum BUN (41.28±1.66; p<0.001***), creatinine (5.66±0.39; p<0.001***), uric acid (72.49±2.60; p<0.001***), increased sodium (80.16±2.85; p<0.001***), chloride (63.66±2.64; p<0.001***), compared to sodium oxalate induced urolithiasis rats (Figure: 5 and 6).

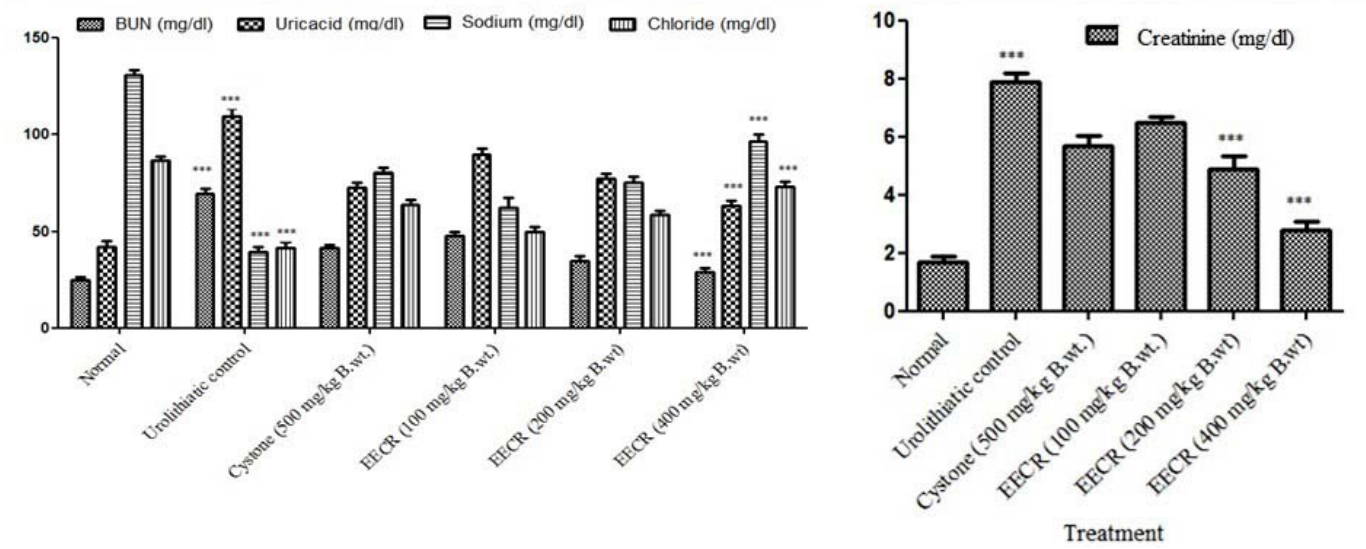

FIGURE 5 - Effect of EECR on urine BUN, uric acid, sodium and chloride in urolithiasis rats.

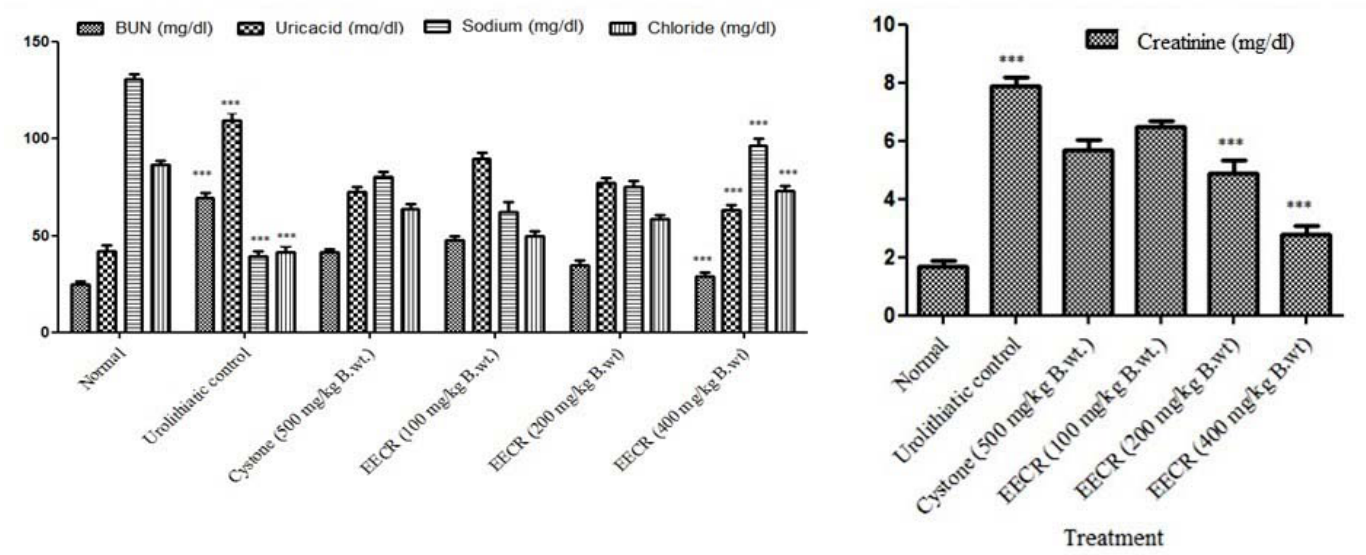

FIGURE 6 - Effect of EECR on urine creatine urolithiasis rats.

DISCUSSION

Ethnobotanical reports revealed that a large number of plants were used for urological problems such as kidney disease, kidney and ureter calculus, dysuria, nocturia, cystitis (Ahmed, Hasan, Alam Mahmood, 2016) Many plants are popular in traditional medicine and used in urolithiasis, only a few studies have been carried out on their exact role, effectiveness and side effects (Gürocak, Küpeli, 2006).In this study was investigated the anti urolithiatic activity of ethanolic extract of *Cyperus rotundus*tubersusing *In silico, in vitro* and *in vivo* rat model.

*Cyperus rotundus*tubersconstituents were subjected to docking studies with Oxalate oxidase, which was found to be one of the responsible factors for stone deposition (Dahiya, Pundir, 2013). In this study was observedHumulene epoxide, 4-Oxo-alpha-ylangene and Cubebol exhibited strong interaction with Oxalate oxidase with lesser docking binding energy. Investigations of the docked conformations of Oxalate oxidase onHumulene epoxide, 4-Oxo-alpha-ylangene and cubebol revealed that

carbon skeleton provides sites for receptor interaction by H-bond and hydrophobic interaction in the binding pocket. This docking work prompted to us to further go for wet experiments with these extracts. The *in vitro* inhibitory effect of EECR on various phases of calcium oxalate crystallization was determined at different concentrations of 50-3200 µg/ml. Calcium chloride and sodium oxalate was used to evaluate the calcium oxalate crystallization. Synthetic urine supersaturated with calcium chloride and sodium oxalate was used to evaluate the calcium oxalate crystallization. It is difficult to mimic the urinary tract *in vitro*, as the normal urine in the human body is not a static solution as new solutes are constantly being added and subtracted from the solution. However, the nucleation and aggregation of crystals in synthetic urine under a static environment helps in explaining the growth of urinary calculi in the body to a certain extent. We have selected a constant p^H of 6.5 as the literature studies revealed a predominance of calcium oxalate monohydrate particles over calcium oxalate dihydrate particles at a p^H5.5–7.0, but this situation was reversed with increasing p^H. At p^H levels above 7.0 not only was there predominance of calcium oxalate dihydrate particles, but also crystal number and diameter were dramatically reduced(Fan *et al.,* 1999; Fan *et al.,* 2001). The levels of urinary supersaturation correlate with the type of stone formed, and lowering supersaturation is effective for preventing stone recurrence (Beghalia *et al.,* 2008).

In the present study, the extract caused an important inhibition of calcium oxalate crystallization during the nucleation phase. Crystalluria alone is not a risk factor for lithiasis, however, as it is common in both healthy people and those who are prone to formation of stone. The limiting factors in urolithiasis could also be those processes that affect the size of the formed particles, as particles can become large enough to overshadow the urinary tract leading to the formation of stones (Beghalia *et al.,* 2008). In this regard, EECR has inhibitory effect on aggregation of calcium oxalate crystals. Furthermore, the decreased number of calcium oxalate crystals due to extract treatment has several positive virtues. Firstly, it shows that substances from the plants exert their action directly or indirectly on crystal morphology. Secondly, the appearance of oxalate dihydrate particles than calcium oxalate monohydrate particles is advantageous since calcium oxalate monohydrate crystals have higher adhesion affinity to renal epithelial cells than calcium oxalate dihydrate particles (Atmani, Khan, 2000). Similar results of inhibition of aggregation of calcium oxalate crystals were also obtained by the *Terminalia arjuna* bark (Choudhary, Singla, Tandon, 2010), *Alismatis rhizome* (Butterweck, Khan, 2009), *Dolichos biflorus* seeds (Saha, Verma, 2015).

A number of animal models with rats were used to induce urolithiasis by calcium oxalate. Sodium oxalate induced urolithiasis is a model that develops rapid calcium oxalate crystals in renal tubules in experimental animals and is therefore commonly used for rapid anti urolithic drug screening. (Thangarathinam *et al.,* 2013). In urolithiasis, the stones in the urinary system interfere with urine outflow, cause decreased glomerular filtration rate and ultimately cause the accumulation of nitrogenous substances such as urea, creatinine and uric acid in the blood and also increase Na^+, Cl^-urinary excretion compared to normal rats (Pawar, Vyawahare, 2017). These changes were inhibited by EECR administration, which reduced serum creatinine, BUN, uric acid and increased urinary Na^+, Cl^-excretion.

Previous studies support the idea that renal tubular injury caused by free oxalate radical production is closely linked to urolithiasis pathogenesis (Khobragade *et al.,* 2011; Dinnimath, Jalalpure, 2015). Hence, it is shown that natural antioxidants are prevented the development of papillary and intratubular calcification of the kidney, It is therefore demonstrated that natural antioxidants are prevented from developing papillary and intratubular kidney calcification, thus preventing the development of papillary calculiin rats with urolithiasis caused by ethylene glycol. (Grases *et al.,* 2009). Studies have shown that terpenes have potent antioxidant effects and reduce the formation of calcium oxalate crystals (Rm *et al.,* 2018; Singh *et al.,* 2010) and also ethanolic extract of *Cyperus rotundus* was showed significant antioxidant activity (Khwaja, Mahmood, Siddiqui, 2016)

*Cyperus rotundus*tubers consist of essential oils such as4-Oxo-alpha-ylangene (12.8%), Humulene epoxide (2.6%)and cubebol (0.5%)(Aghassi, Naeemy, Feizbakhs, 2013; Al-Massarani *et al.,* 2016) owing to

elevated antioxidant activity (Yazdanparast, Ardestani, 2007; Kilani *et al.,* 2008; Singh *et al.,* 2010) may be suggested as the primary active values with preventive impact in urolithic rats.

CONCLUSIONS

In the present study, an attempt was made to investigate the protective effect of medicinal plants against urolithiasis by means of molecular docking with *Cyperus rotundus* case studies and to further validate its results experimentally. In our preliminary study, antiurolithic activity extract was demonstrated in nucleation, aggregation test and sodium oxalate-induced animal models of urolithiasis. In order to understand its interactionwith oxalate oxidase binding protein, structures of all the reported terpene metabolites present in both theextractswere subjected to *in silico* molecular dockingto obtain biological activity spectrum of each component. Our study concludes that these compounds will speed up the discovery of possible leads from the natural medicinal plant in the prevention of nephrolithiasis.

CONFLICT OF INTEREST

Authors no conflict of interest.

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