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Insilico And Invitroevaluation Of Anti-Urolithiatic Activity Of Ethanolic Extract Of Hybanthus Enneaspermus (Linn.) F. Muell.,

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ABSTRACT: Objective: The term urolithiasis is the formation stones in urethra/bladder of urinary system. The herbal plant Hybanthus enneaspermus (Linn.) F. Muell., is used for inhibition, prevention and for treatment of urinary calculiin order to lessen the side effects of allopathic medicines.The main objective of this study to investigate anti-urolithiatic property of Hybanthus enneaspermus (Linn.) F. Muell by insilico and invitro methods. To assess the effect of plant against urolithiasis in vitro study was conducted with ethanolic extract and standard drug, Cystone.

Material and Methods: In this study, five assays such as crystal dissolution, crystallization, nucleation, aggregation and growth assay of crystal were performed. In silico studies were done by using AutoDock 4.2 tools to determine the binding energy and affinity of chemical constituents (Rutin and Quercetin) against the targets Adenine phosphoribosyltransferase, Oxalate oxidase, Glycolate oxidase with their Pdb id: 1L1Q, 2ETE, 2RDT.

Results: The results prove the significant activity of ethanolic extract of Hybanthus enneaspermus against urolithiasis. when compared with cystone,the plant extract has the capacity to prevent and inhibit the crystal dissolution, crystallization, nucleation, aggregation and growth of crystal. On the other side Hybanthus enneaspermus showed strong binding energy and affinity towards the targets Adenine phosphoribosyltransferase, Oxalate oxidase, Glycolate oxidase in insilico studies.

Conclusion: This study proved that the Ethanolic extract of Hybanthus enneaspermus has a potent and therapeutic urolithiatic activity.

Keywords: Urolithiasis, Adenine phosphoribosyltransferase, Oxalate oxidase, Glycolate oxidase.

I. INTRODUCTION:

In the total world population, approximately 85% use the herbal or traditional medicines in health-related problems. In the recent years therefore, high development in research area which has been focused on evaluation of herbal medicines from plants scientifically. This era of research on traditional drugs has created the belief of safe and no side effects motivated humans to return to natural remedies when compared to synthetic drugs which have lots of side effects[1]. Urolithiasis is a process of forming stones in the kidney, bladder, and/or urethra (urinary tract)[2]. In urinary system, urolithiasis (Greek- ouron, "urine" and lithos, "stone") is the condition where urinary stones are formed or located. Urinary stones are firm, dense particles that produced in the urinary system[3]. If large amount of stone produced, it may block the urine flow result in agonizing pain. Patient with urinary stones with continuous medical care have common recurrent stone formation. Some synthetic drugs itself results in urinary stones[4]. There are various types of stones present in urinary stones with composition of calcium, phosphate, sodium, magnesium etc. 80-90% of Calcium stones occurs in most of the cases followed by struvite stones 15-20% then uric acid stones 5-10% and least with 1% of cystine stones are seen[5].Hybanthus enneaspermus commonly known as Spade flower belongs from the family violaceae widely distributed in India, Srilanka, Malaysia, Africa, South East China to tropical Australia used for the treatment of diarrhoea, urinary infections, leucorrhea, dysuria, inflammation, cholera, sterility, vomiting, burning sensation, blood troubles, asthma, epilepsy and breast tone[6,7].To assess the effect of Hybanthus enneaspermus against urolithiasis in vitro study was conducted with ethanolic extract and standard drug, Cystone. Molecular docking is one of major computer assisted drug design for determining the affinity of

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newly discovered drugs with the targeted enzymesi.e. Ligand-protein docking[8]. By review of literature, somecompounds which are isolated through various solvent is identified through analysis procedures and the structures of the ligands can be docked with the protein intricate in pathway of urolithiasis[9].The aim of the current study was to investigate the Insilico and invitro anti-urolithiatic activity of ethanolic extract of Hybanthus enneaspermus (Linn.) F. Muell.

II. MATERIAL AND METHODS

In-silico docking studies[10]:

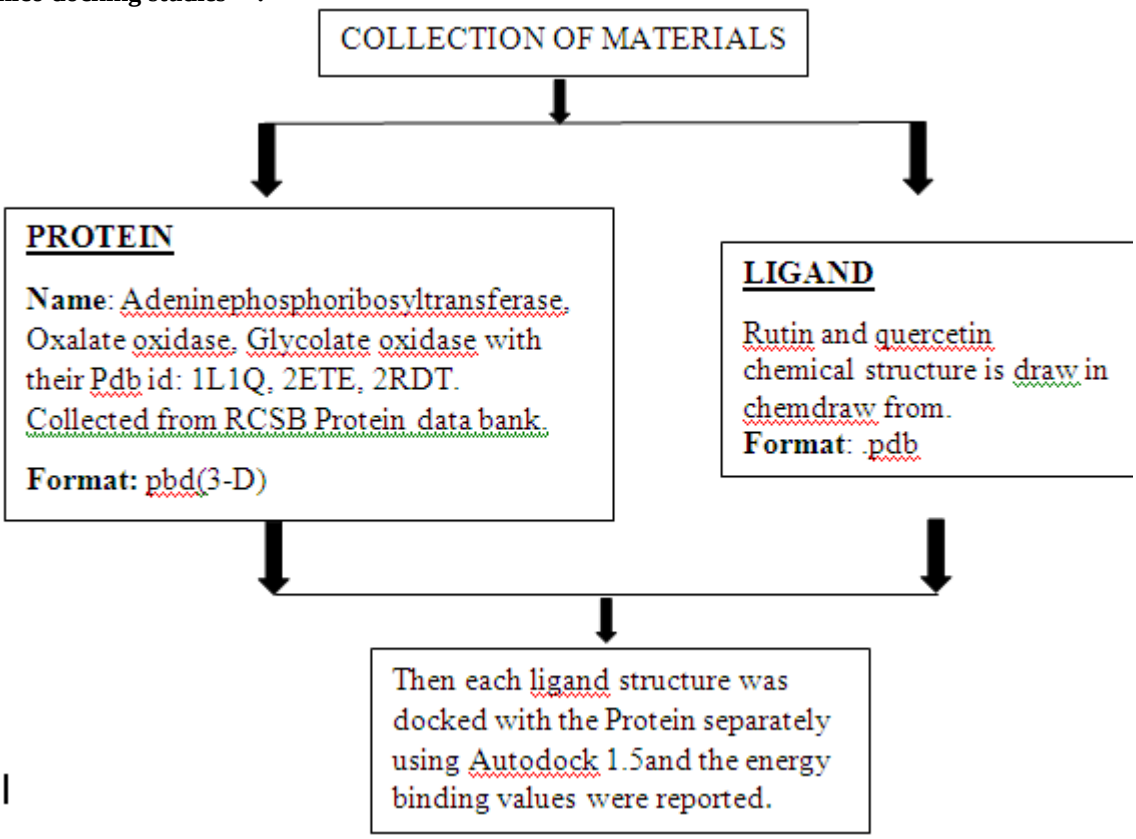

Fig 1: Protein and Ligand used in Insilico Studies.

Thecompounds choose for the docking studies is based upon the review of literature. The ethanolic extract of Hybanthus enneaspermus is used for isolation of compounds and based upon the activity of compounds the ligands were selected with their chemical structures and those were used to bind with target to determine the binding affinity for the docking studies[11]. Identified compounds were studied by using auto-mated docking software (Autodock 1.5). The protein sequence of Adenine phosphoribosyltransferase (APRTase), Oxalate oxidase and Glycolate oxidase with two molecule Rutin and Quercetin[12].

Plant collection:

The plant of Hybanthus enneaspermus was collected from local place in Chennai, Tamilnadu (INDIA), in the month of June. It was authenticated by Prof. V. JAYARAMAN, Director, Professor (Rtd), Presidency college Chennai-5.

Plant Extraction

The whole plants were rinsed and mince into small pieces. It is dried under shade and pulverized. Extraction is done by soxhlet apparatus by using ethanol as solvent. The obtained material was heated in on a water bath until residue was obtained and then dried in a desiccator[13].

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In-vitro Anti- Urolithiatic activity:

Calcium Oxalate Assay by Titrimetric Method[14]

In this method, the feigned prepared calcium oxalate crystal wastaken in semipermeable membrane of egg which taken as control and then plant extract and standard Cystone were taken separately and added to semipermeable membrane. Further it was submerged in Tris buffer solution and incubated for 2 hours at 37℃. Removal of semi permeable membranes content after 2hours and 2ml of 1N sulphuric acid added titrated against potassium permanganate till end point of light pink colourwas obtained.

The calcium oxalate assay by titrimetric method involved three steps.

1. Preparation of experimental calcium oxalate stones by precipitation method.
2. Preparation of semi-permeable membrane from eggs.
3. Evaluation of calcium oxalate by Titrimetry assay method.

Preparation of experimental calcium oxalate stones by precipitation method:

Calcium oxalate crystals prepared byhomogenous precipitation method. 1.47gm of calcium chloride dihydrate and 1.34gm of sodium oxalate wasdissolved in 100 ml distilled water and 100 ml of 2N H2SO4 respectively. The precipitated calcium oxalate is obtained whenthe two contents were assorted equally in a beaker. The resultant calcium oxalate was exposed to ammonia solution to free from sulfuric acid traces,then washed with distilled water, dried for 2 hours at 60°C.

Preparation of the semi-permeable membrane from eggs

The apex of eggs was punctured using a glass rod to remove the entire content. The egg empty shell was washed with distilled water and set down in a beaker consisting 4ml concentrated HCl in 200ml distilled water. Decalcification occurs, when semipermeable membrane of egg is kept for overnight.The very next day, the semi permeable membranes are detached carefully from the egg shells and washed with distilled water and to neutralize the acid traces ammonia solution is also used and then cleanse with distilled water. It was stored in refrigerator at Ph of 7-7.4 in moistened condition.

Fig 19: Decalcification of eggshell in 10% contents acetic acid overnight.

Fig 20: Egg membrane along with the suspended into the 0.1 M Tris buffer.

Evaluation of calcium oxalate by Titrimetry assay method:

1 mg of calcium oxalate packed in egg semipermeable membrane added to 100 ml0.1M Tris buffer act as control.1mg of calcium oxalate with 10 mg of ethanolic plant extract as test, 10 mg of cystone as standard packed with egg semipermeable membrane and drape in a beaker containing 100 ml of 0.1M Tris buffer. All the samples containing semipermeable membrane were incubated for 2 hours at 37℃. The contents of semipermeable membraneare removed into separate test tube at different concentrations of standard drug and ethanolic plant extract. 2ml of 1N sulphuric acid added to each test tube and titrated against0.9494N potassium permanganate till end point of light pink colourwas obtained.The total quantity of dissolved calcium oxalate is obtained by the amount of remaining undissolved calcium oxalate is subtracted from the total

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quantity used in the experiment.The percentage dissolution of calcium oxalate in various groups was calculated as:

Dissolved calcium oxalate = (Undissolved calcium oxalate) – (Total quantity used in the Experiment in the beginning)

Percentage dissolution = Dissolved calcium oxalate X 100.

In Vitro Calcium Oxalate Crystallization Inhibition[15]:

In vitro anti-urolithiatic activity Hybanthus enneaspermus of whole plant extract were tested in terms of inhibition of calcium oxalate formation by the extracts in the presence (standard drugs and extract) and absence of inhibitors.The calcium oxalate precipitation in pH 6.8 and at 37℃ is done by turbidity measurement using UV/Vis spectrophotometer at 620nm. It was working to estimate the turbidity of calcium oxalate.

Study without inhibitor

1ml of 0.025M sodium oxalate were added to 1 ml of 0.025M calcium chloride dihydrate and 2 ml of Tris-buffer (pH7.4)in a test tube.Measure the turbidity up to the period of 10 minutes after mixing of above solutionby UV/Vis spectrophotometer at 620nm. The solution is taken as control and the experiment was done in three replications.

Study with inhibitor

1ml of 0.025M sodium oxalate were added to 1 ml of 0.025M calcium chloride dihydrate and 2 ml of Tris-buffer (pH7.4)in a four sets of test tubes and 1ml (10 mg/ml solution) of ethanolic plant extracts were added, same procedure is repeated with standard drug poly herbal formulation cystone in place of plant extract. Measure the turbidity up to the period of 10 minutes after mixing of above solutionby UV/Vis spectrophotometer at 620nm. Each step is repeated three times.The % of inhibition was calculated using the formula:

I (%) = [1-Ti/ Tc] x 100

Where,

Ti is turbidimetric slope with inhibitor,
Tc is turbidimetric slope without inhibition.

Nucleation Assay[16]

Precipitation of crystals formed from Solutions of calcium chloride and sodium oxalate at a final concentration of 5 mmol/L and 7.5 mmol/L, respectively, then add in a buffer containing Tris 0.05 mol/L and sodium chloride 0.15 mol/L at pH 6.5; by taking 0.22 µm filterboth solutions were filtered 3 times. Then 100 µl each of ethanolic extract and standard cystone at different concentrations (100, 200, 400, 800, and 1000 µg/ml) were mixed with 950 µl of calcium chloride solution. 950 µl of sodium oxalate solution was added with occurance of crystallization. Solution was stirred magnetically at 800 rpm for 15 min, and maintained at 37°C. The measurement of optical density of the crystallized suspension takes place inspectrophotometer at 620 nm after addition of calcium-containing solutions. The whole experiments were repeated in triplicate.In the presence of plant extract and cystone was compared with control to determine percentage inhibition.

Percentage Inhibition = {(OD control-OD sample)/OD control} ×100

Where, OD control - Optical density of control

OD sample - Optical density of sample

Aggregation Assay[17]:

Calcium chloride solution 50 mmol/L and sodium oxalate of 50 mmol/L added together to prepare calcium oxalate monohydrate crystals. In a water bath for 1 h the solution was equilibrated to 60°C andcooled to 37°C overnight. By centrifugation the crystals were harvested and at 37°C it was evaporated. Then buffering of calcium oxalate crystals with0.5 ml of 0.15 mol/L NaCl solution and 0.5 ml of 0.05 mol/L Tris bufferat pH 6.5 to a finalconcentration of 1 mg/ml. In the absence or presence of ethanolic extract andcystonethe experiments were conducted at 37°C at different concentrations (100, 200, 400, 800, and 1000 µg/ml) with constant stirring. The optical density was measured at 620 nm in a spectrophotometer after stopping the stirringand the percentage inhibition of aggregation was determined using the same formula as in turbidity method.By comparing the turbidity in the presence of the extract with that obtained in the control,the percentage aggregation inhibition rate (Ir) was calculated using following formula

Ir = (1−Turbiditysample/Turbidity control) × 100

Where, **Ir** is the percentage aggregation inhibition rate.

Growth Assay[18]

The relative percentage inhibition was evaluated in presence and absence of plant extracts

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using calcium oxalate crystal growth assay method. 1 ml of 4mM sodium oxalate and 1 ml of 4mM calcium chloride each were added to a 1.5ml solution of10mMsodium chloride and then buffered with 10mM Trisat pH 7.2. Calcium oxalate monohydrate crystal slurry (1.5mg/ml acetate buffer) about 30 µl was added tothe obtained solution. Utilization of oxalate begins at once after COM slurry addition and was observed for 30 minutes for vanishing of absorbance at 214nm.Thestandard drug and were individually added into the reaction mixture. The standard drug (cystone 1000 µg/ml) and plant extract was taken in different concentrations (Plant extract 400, 800,1000 µg/ml respectively). If extract inhibits calcium oxalate crystal growth the depletion of free oxalate ions will be decreases. The relative inhibitory activity wasdetermined by the following formula:

Percentage Relative inhibitory activity = ((C-S)/C) × 100

Where,

C - rate of reduction of free oxalate without any extract

S - rate of reduction of free oxalate with drug extract.

III. STATISTICAL ANALYSIS

The data obtained were analyzed by one way ANOVA followed by student's t-test using the SPSS software wherever necessary. A value of $p < 0.05$ and $p < 0.01$ was considered as highly significant and significant in all cases.

IV. RESULTS AND DISCUSSION:

The shade dried entire plant of Hybanthus enneaspermus was extracted in soxhlet apparatus successively with ethanol. The percentage yield was calculated for the extract in terms of dried weight of plant material. The colour and consistency of the extract are given in table no. 1.

S.No	Extract	Method of Extraction	Physical Nature	Colour	Yield (%W/W)
1.	**Ethanol**	Continuous Hot percolation method using Soxhlet apparatus	Semisolid	Dark brown	1.68

Table 1:Percentage yield of ethanolic extract of Hybanthus enneaspermus

Insilico docking analysis:

Best Interaction Docking Pose of various Chemical Compounds withAdenine Phosphoribosyltransferase:

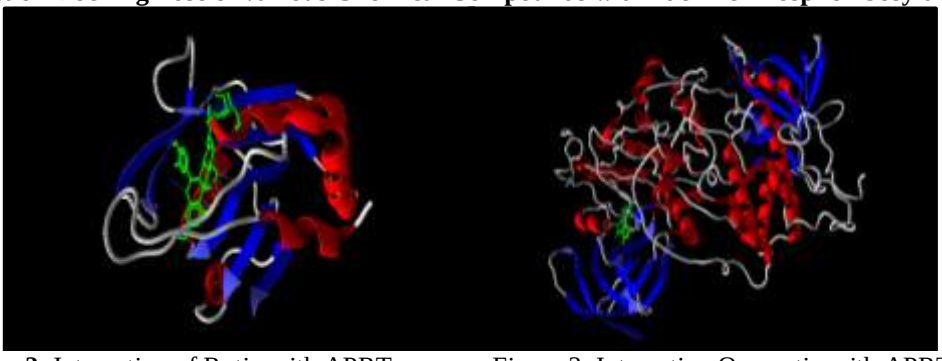

Fig no 2: Interaction of Rutin with APRTase Fig no 3: Interaction Quercetin with APRTase

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Best Interaction Docking Pose of various Chemical Compounds with Oxalate oxidase:

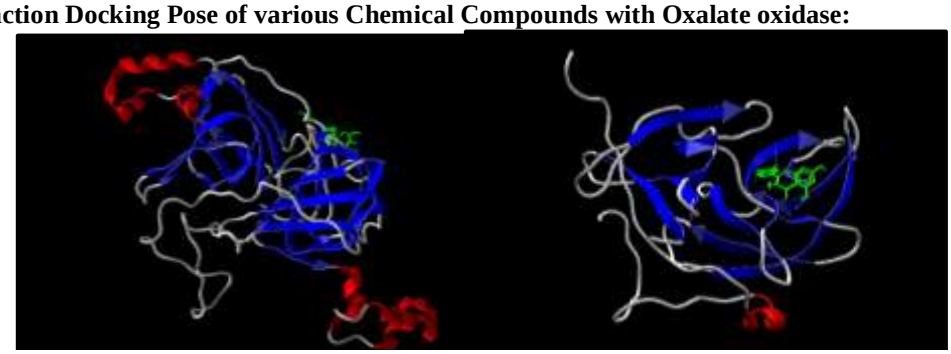

Fig 3: Interaction of Rutin with oxalate oxidase Fig 4: Interaction Quercetin with oxalate oxidase

Best Interaction Docking Pose of various Chemical Compounds with glycolate oxidase:

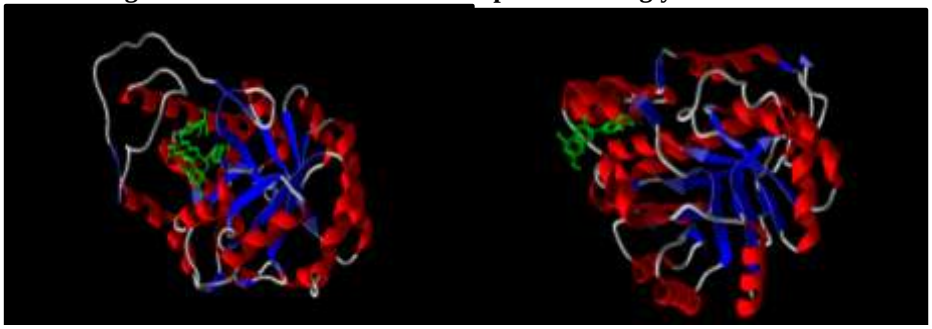

Fig 5: Interaction of Rutin with glycolate oxidase Fig 6: Interaction Quercetin with glycolate oxidase

S.no	Target	Binding energy ofRutin KJ (mol-1)	Binding energy ofQuercetin KJ (mol-1)
1.	AdeninePhosphoribosyltransferase	-11.66 kJ mol-1	-9.90 kJ mol-1
2.	Oxalate oxidase	-11.28 kJ mol-1	-7.62 kJ mol-1
3.	Glycolate oxidase	-12.01 kJ mol-1	-10.84 kJ mol-1

Table 2: The Docking results ligands showed binding energy with proteins.

IN VITRO STUDIES

Table3: Effect of Hybanthusenneaspermus on percentage dissolution of calcium oxalatecrystals

S.NO	Experimental design Concentration (µg/ml)	Dissolved calcium oxalate (mg)	Percentage (%) dissolution
1.	Control	0.10	10
2.	Cystone 1000 µg/ml	0.87	87
3.	HEEextract 200 µg/ml	0.56	56
4.	HEE extract 400 µg/ml	0.69	69
5.	HEE extract 800 µg/ml	0.75	75
6.	HEE extract 1000 µg/ml	0.86	86

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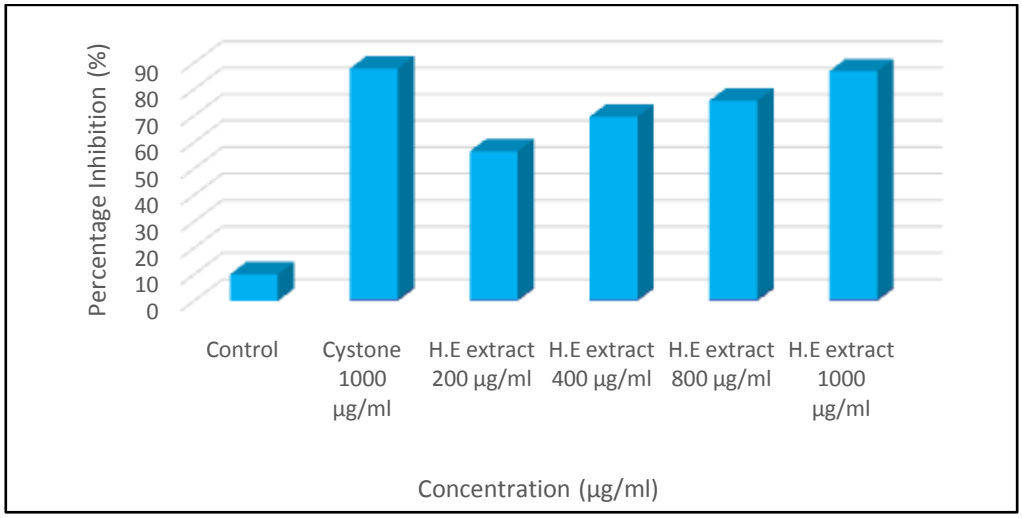

Fig7: Effect of Hybanthus enneaspermus ethanolic extract and cystoneon calcium oxalate crystals by titrimetric method.

S.no	Concentration (µg/ml)	% Inhibition	
		Ethanolicextract	Cystone Standard
1	200	38.30±0.264**	37.00±0.100**
2	400	53.50±0.173**	52.66±0.251**
3	600	54.83±0.550	54.16±0.450
4	800	66.10±0.200**	67.77±0.591**
5	1000	80.54±0.115**	81.24±0.488**

Table4: The percentage inhibition of Hybanthus enneaspermus whole plant extracts atdifferent concentrations.

Values are expressed as Mean±SEM (n=3)
Symbols represents statistical significance ***-P<0.001, **-P<0.01, *-P<0.05.

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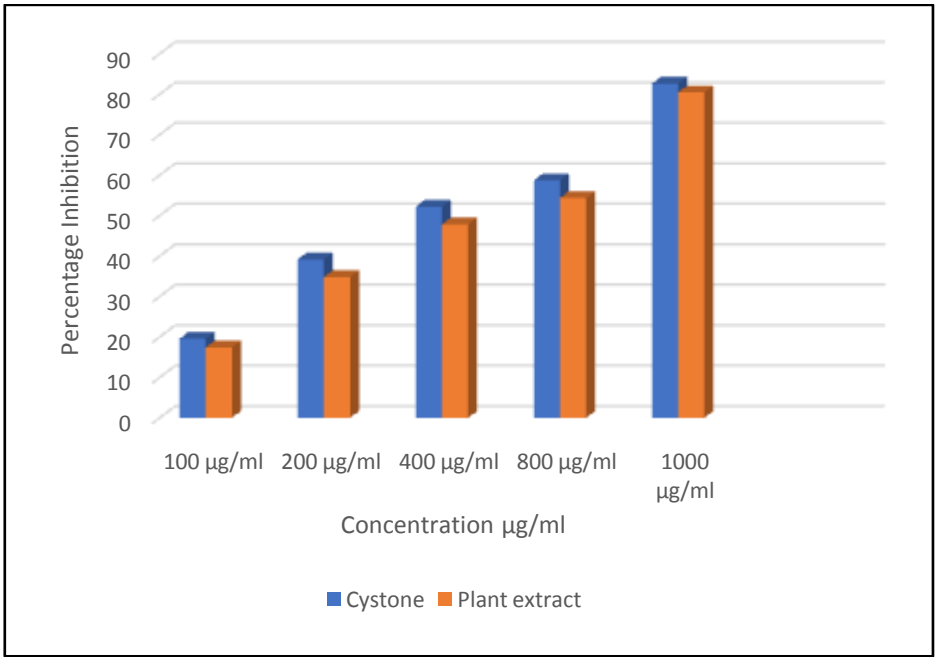

Fig8: Graphical representation of calcium oxalate crystallization inhibition

Table5: Inhibitory effect of Hybanthus enneaspermus on nucleation assay

S.no	Concentration (µg/ml)	Ethanolic Extract (Optical density)	% Inhibition	Cystone (Optical density)	% Inhibition
1.	100	0.38±0.02	17.39	0.37±0.03	19.56
2.	200	0.30±0.01**	34.78	0.28±0.02**	39.13
3.	400	0.24±0.03**	47.82	0.22±0.05**	52.17
4.	800	0.21±0.05**	54.34	0.19±0.05*	58.69
5.	1000	0.09±0.02**	80.43	0.08±0.02**	82.60

The optical densities were expressed as mean±SD; **p<0.01 was considered as significant when compared with control OD = 0.46±0.01.

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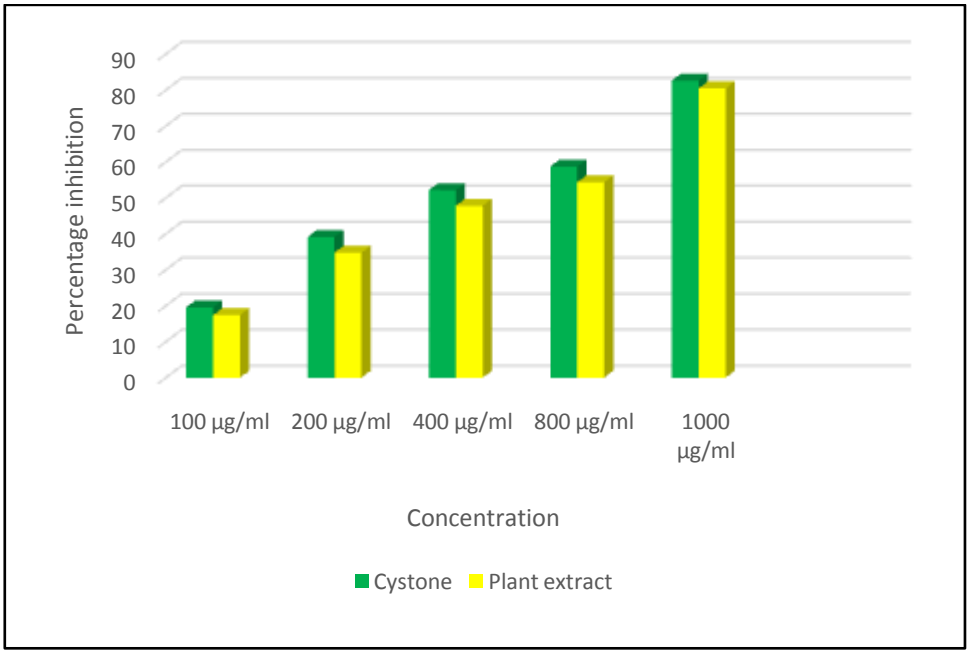

Fig9: Effect of Hybanthus enneaspermus plant extract on nucleation of crystals

Table6: Inhibitory effect of Hybanthus enneaspermus on aggregation assay

S.no	Concentration (µg/ml)	Ethanolic Extract (Optical density)	% Inhibition	Cystone (Optical density)	% Inhibition
1.	100	0.42±0.021**	20.75	0.40±0.023**	24.52
2.	200	0.35±0.080	33.96	0.27±0.080	49.05
3.	400	0.25±0.026**	52.83	0.22±0.026**	58.49
4.	800	0.17±0.023*	67.93	0.20±0.021**	62.27
5.	1000	0.10±0.013**	81.01	0.09±0.015**	82.02

The optical densities were expressed as mean±SD; **p<0.01 was considered as significant when compared with control OD = 0.53±0.01.

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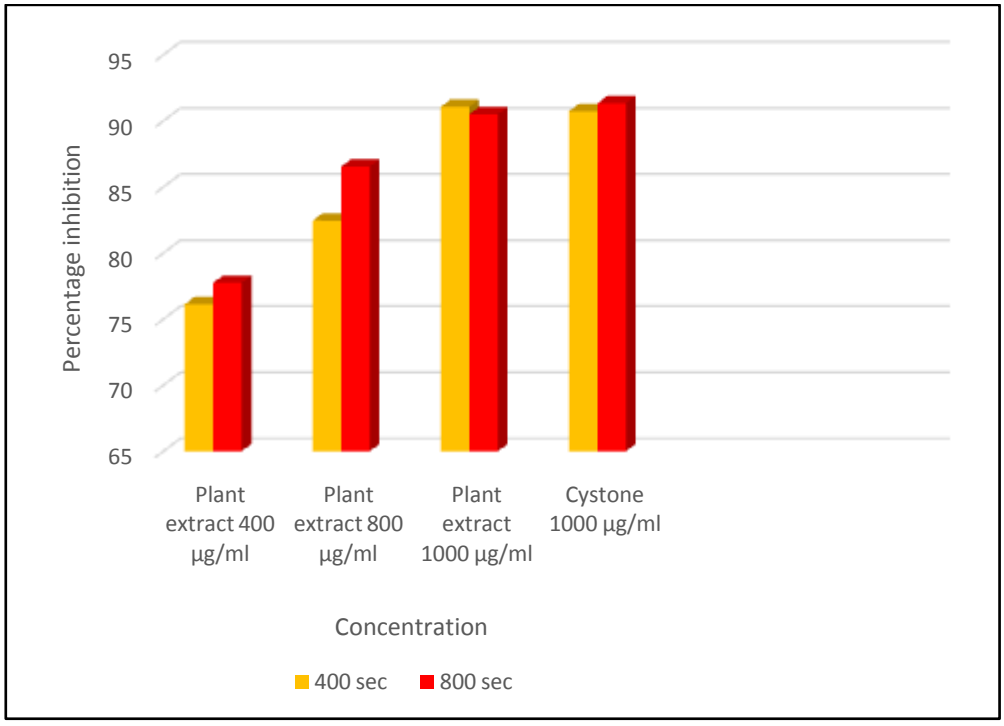

Fig 10: Effect of Hybanthus enneaspermus plant extract on crystal aggregation

Table 7: Effect of Hybanthus enneaspermus on growth assay

S.no	Concentration (µg/ml)	% Reduction of free oxalate			% Relative inhibitory activity {% RI = [(C-S)/C] × 100}	
		400 sec	800 sec	8008	400 sec	800 sec
1.	Control	6.16±0.45	5.80±0.33		-	-
2.	Cystone (1000µg/ml)	0.57±0.05	0.55±0.05		91.74	91.34
3.	Plant extract (400 µg/ml)	1.47±0.1	1.29±0.09		76.13	77.75
4.	Plant extract (800 µg/ml)	1.08±0.04	0.78±0.09		82.46	86.55
5.	Plant extract (1000 µg/ml)	0.55±0.06	0.56±0.05		91.07	90.51

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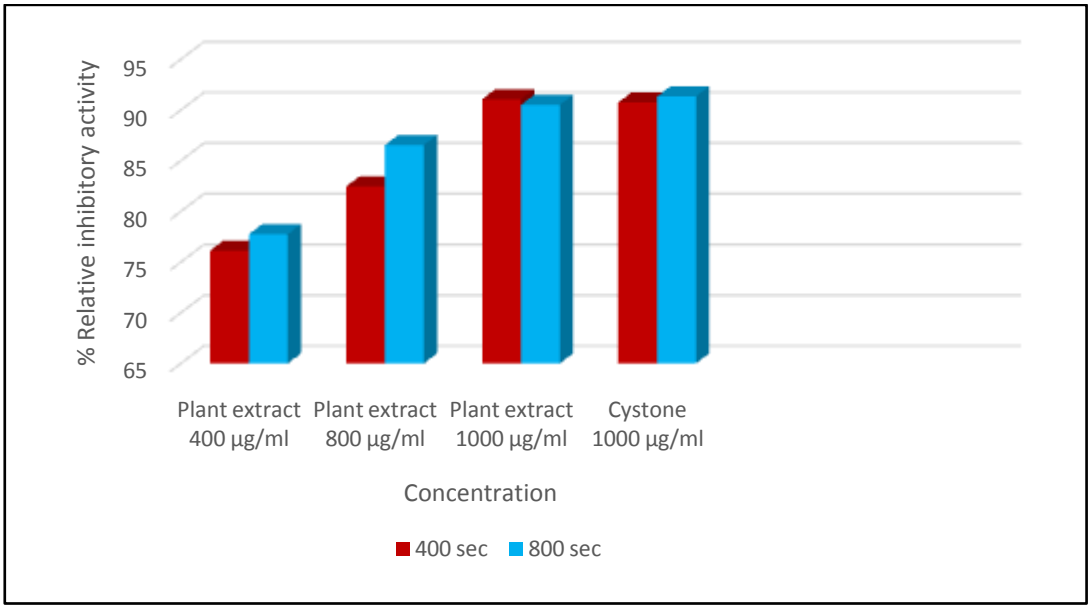

Fig11: Effect of Hybanthus enneaspermus plant extract on crystal growth

Urolithiasis, (stone formation) is an important cause for acute and chronic renal failure, includes bothnephrolithiasis (stone formation in kidney) and urolithiasis (stone formation in ureter or bladder or both)[19]. Various stone which was identified mostly occur in men for e.g. calcium stones while phosphate stones formation is more in women.The percentage of successive extractive value for the entire plant of Hybanthus enneaspermus Linn., (F) Muell. is tabulated in Table 1. Percentage yield of ethanolic extracts were 1.7% of yield.Hybanthus enneaspermusus extract acts by inhibiting XDH, thus preventing 2,8-dihydroxyadenine synthesis[20].After molecular docking both the compound have binding affinity towards the adenine phosphoribosyltransferase but Rutin (-11.66 kJ/mol) was observed high binding free energy with hydrogen bonds Tyr 101, Glu 61, Leu 28, Lys 26 and Quercetin (-9.90 kJ/mol) observed less.The docking of legend molecules with 2 ETE of Oxalate oxidase which reportedly participate in kidney stone formation in patients, reveals that both the inhibitor compounds exhibited the binding with one or other amino acids with better regio-specificity in the active pockets[21]. Among the tested compounds, docking of 2 ETE with rutin revealed that binding energy of -11.28 kJ/mol with hydrogen bonds Asn 167, Gly 87, Trp 83, Asn 152, Ala 164, Leu 149 isfavourable for a good inhibitor of oxalate oxidase.Glycolate oxidase (GOX) in abnormal condition catalyzes the FMN-dependent (Flavin mononucleotide) oxidation of glycolate to oxalate, a key metabolite leading to increased excretion of oxalate which results in to the deposition of kidney stones[22].The binding energy of rutin and quercetin 12.01 kJ/mol with hydrogen bonds Glu 280, Val 288, Gly 307, Lys 309, Leu 304 and 10.84 kJ/mol respectively. Rutin have higher binding affinity when compared to quercetin and good inhibitor of glycolate oxidase.The ethanolic extract of Hybanthus enneaspermuswhole plant showed 86percent dissolution of calcium oxalate at concentration 1000 µg/ml. While Cystone a prescribed medicine for renal calculi showed highest percentage dissolution (87%) of calcium oxalate by the Titrimetry method. Therefore, the ethanolic extract of Hybanthus enneaspermus were almost significant to that of cystone (standard) and proves that plant possess both inhibitory as well as therapeutic effects in urolithiasis.In Vitro Calcium Oxalate Crystallization Inhibition study clearly indicates that the ethanolic extract of Hybanthus enneaspermuswhole plant showed higher calcium oxalate crystallization inhibition (80.54 %) at 1000 µg/ml concentrationalmost significant to that of standard drug. Ethanolic extract and standard cystone were very significant ($p<0.01$)capability to dissolve calcium oxalate as foremost element for stone forming in the urinary tract.The nucleation assay was done by turbidity method, and nucleation of crystals was determined by a nucleation assay.Hybanthus enneaspermusethanolic extract showed potent inhibition at 1000 µg/ml when compared to the standard drug cystone.The maximum percentage inhibition of crystal

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aggregation by ethanolic plant extract and cystone were found to be respectively 81.01%, 82.02% at 1000 µg/ml. Hybanthusenneaspermus extract and standard cystone very significantly ($p<0.01$) inhibited the nucleation and aggregation of urinary crystals.The percentage relative inhibitory were higher in plant extract (91.07%) at 1000 µg/ml in 400 seconds nearer to standard drug cystone (91.74%) shows the potent inhibitory activity of plant.

V. CONCLUSION:

The molecular docking analysis is clearly showing that the compounds (Rutin and Quercetin) functionally active to interrupt the various pathways of enzymes and prevent the urinary stone. The results obtained from insilico docking studies showed an effective inference on utilization of the compounds showed higher binding energy and affinity towards the enzymes and used as treating aids for urolithiasis.The results clearly indicate that, under in vitro conditions, ethanolic extract were found to have inhibitory effect on calcium oxalate crystallization and also express a concentration-dependent inhibition on the crystal nucleation, aggregation and crystal growth. The ethanolic extracts were also having more capacity to dissolve the already formed urinarystones. These findings substantiate the traditional use of Hybanthus enneaspermus in the treatment of urinary stones and kidney problems.To substantiate itsinvitro effect,further investigations forinvivo studies need to be carried out in experimental animals, then this drug can be used for the treatments of urinary and kidney stones.

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