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Mechanistic Insights into the Anti-Urolithiatic Potential of *Ecbolium viride* Ethanolic Extract: *In Silico* Targeting of Human Glyoxylate Reductase Hydroxypyruvate Reductase and Adenine Phosphoribosyltransferase, Supported by *In Vitro* Validation

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Abstract

Urolithiasis is characterized by the formation of urinary stones and represents a significant global health burden, frequently necessitating invasive treatments and leading to recurrent symptoms. Due to the limitations of conventional therapies, there is a growing interest in alternative treatments, especially those based on traditional herbs. This research examines the therapeutic potential of *Ecbolium viride* leaves for urolithiasis by conducting a thorough evaluation that encompasses phytochemical screening, GC-MS analysis, *in silico* molecular docking, and *in vitro* assays for anti-urolithiasis. Phytochemical screening demonstrated the presence of bioactive compounds, such as alkaloids, flavonoids, tannins, and polyphenols, which are acknowledged for their pharmacological properties. GC-MS analysis further characterized these phytoconstituents, identifying compounds with potential anti-urolithiasis activity. *In silico* molecular docking studies revealed significant binding affinities between these compounds and target proteins associated with stone formation, indicating their potential to disrupt the molecular mechanisms of urolithiasis. *In vitro* assays, such as nucleation, aggregation, and dissolution studies, demonstrated the extract's substantial ability to inhibit the formation and aggregation of calcium oxalate crystals, as well as to enhance crystal dissolution. The results from these methodologies provide substantial evidence that *Ecbolium viride* leaves may serve as a natural agent for managing urolithiasis. This research endorses the application of plant-derived compounds as substitutes for traditional treatments of urolithiasis. Further *in vivo* studies and clinical trials are necessary to confirm the safety, efficacy, and pharmacokinetics of *Ecbolium viride* in the treatment of urolithiasis.

Keywords: Urolithiasis, *Ecbolium viride*, Phytochemical screening, GC-MS analysis, Molecular docking, *In vitro* assays, Calcium oxalate crystals, Anti-urolithiasis activity

Introduction

Urolithiasis, or kidney stones, is a common health problem around the world. Biochemical imbalances in urine produce kidney or urinary tract stones. Pain, hemorrhage, and renal failure can result from these stones. Therefore, preventing and addressing this issue is vital [1]. At 5 - 15 % prevalence, it costs the US about 2 billion dollars in healthcare annually, according to study. Obesity, diabetes, salt intake, and climate change enhance stone development. The lifetime risk of kidney stones is 12 % for males and 6 % for women. Recurrent kidney stones increase the risk of ESRD and all-cause mortality. [2,3] Calcium oxalate stones, which form in urine due to dehydration or high oxalate ingestion, are the most common. High calcium and phosphate levels from metabolic illnesses like hyperparathyroidism create calcium phosphate stones. Kidney stone formation is primarily caused by super saturation, crystal nucleation, and growth [4-7]. Stone super saturation occurs when calcium and oxalate ions are high. This will cause crystal nucleation. Osteopontin, a protein, can help crystals adhere and aggregate, but macrophage-driven inflammation, also, oxidative stress promotes stone formation. Acidic urine from hyperuricosuria and low pH causes uric acid stones. Urease-producing bacteria cause urinary tract infections, alkaline urine, and struvite stones. Cysteine stones result from tubule cysteine reabsorption problems. Changes in epigenetic, gene expression, and gut microbiota alter ion handling and oxalate breakdown, increasing stone risk. Stone formation involves genetic, metabolic, inflammatory, and microbiologic factors. Crystal retention and growth are also caused by inflammation and oxidative damage [8-12]. Hydration, food, and drugs treat urolithiasis. Fluid ingestion dilutes urine, causing stone formation and small stone passage. Medication depends on stone kind and location. Potassium citrate is the most frequent urine alkalizer. This dissolves uric acid stones and prevents calcium oxalate [13-18]. Diuretics like hydrochlorothiazide diminish calcium excretion, preventing calcium-based stones. Alpha-blockers like tamsulosin relax urinary tract muscles, making stones easier to pass. [19-21] larger stones may require ureteroscopy, shockwave lithotripsy, or percutaneous nephrolithotomy. Recurrence and negative effects persist despite these therapies, therefore herbal medicine is gaining popularity. Chancapiedra *(Phyllanthus niruri)* reduces calcium oxalate crystallisation; coriander *(Coriandrum sativum)* has diuretic and anti-inflammatory properties; horsetail *(Equisetum arvense)* has silica, which may dissolve stones and prevent crystals. The Acanthaceae family is renowned for its diverse therapeutic characteristics, and a number of plants within it exhibit substantial antiurolithiatic activity. The potential of plants like *Andrographis paniculata*, *Barleria prionitis*, and *Hygrophila spinosa* to prevent the formation of kidney stones has been extensively investigated. These herbs need additional controlled clinical trials to prove their safety and efficacy [22-25]. *Ecbolium viride*, a traditional medicinal herb, possesses anti-inflammatory, antioxidant, and diuretic properties. Despite its widespread use in traditional medicine, its urolithiasis prevention and treatment potential is untapped [26]. *Ecbolium viride* leaves' bioactive compounds may inhibit crystal formation, reduce oxidative stress, and increase urine flow, thereby minimizing stone formation and facilitating breakdown. This study shows how important *Ecbolium viride* is as a possible ingredient for making new medicines to treat urolithiasis. This is shown by its phytochemical profile, GC-MS analysis, and the fact that it works well both *in silico and in vitro.* The found bioactive substances may facilitate stone prevention by suppressing crystal formation.

Materials and methods

Collection and authentication of plant material

Ecbolium viride leaves were gathered from the natural areas around Aravakurichi with the local consent and in accordance with institutional, national, and international standards, such as the IUCN Policy Statement and CITE rules. After species confirmation, the Botanical Survey of India in Coimbatore has kept a voucher specimen (BSI/SRC/5/23/2024/Tech-12) for future use. To remove any microbiological contamination, the leaves were thoroughly rinsed in filtered water before being treated with a 10 % potassium permanganate solution. They were then ground into a fine powder for later use and allowed to dry in the shade.

Preparation of extract

The powdered leaves underwent Soxhlet extraction using ethanol and water in a 70:30 ratio. Following extraction it was then evaporated by rotary evaporator at 40 - 50 °C under decreased pressure. The concentrated extract was then filtered via Whatman filter paper to eliminate any leftover solid particles. Finally, the extract was collected, sealed in appropriate containers, and stored in a cool, dry location for future examination [27].

Preliminary phytochemical analysis

The preliminary phytochemical analysis was performed using the Khandelwal technique standard methodology to detect the numerous secondary metabolites [28-35].

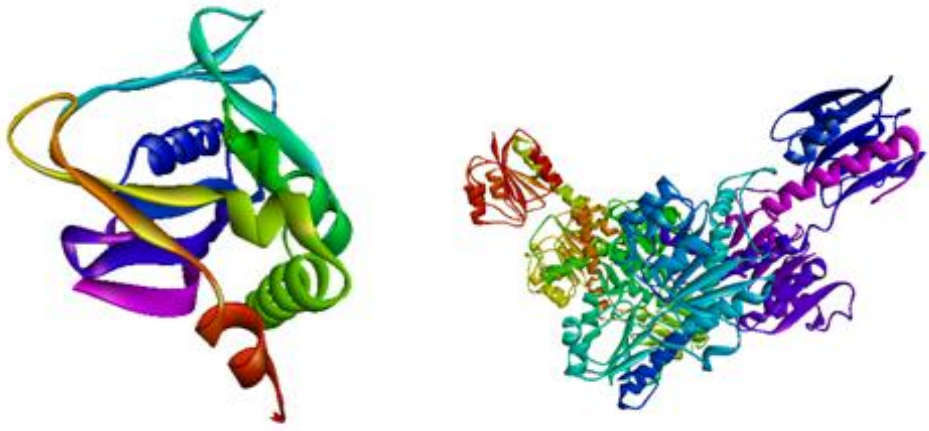
GC-MS analysis

The hydro alcoholic extract of *Ecbolium viridae* was analysed for its phytochemical composition by Shimadzu 17A gas chromatography (GC) system equipped with a Shimadzu QP2010 Plus mass spectrometer. The electron ionisation (EI) technique was used on a 30 m × 0.25 mm i.d., 0.25 µm DB-5 fused silica column in the GC-MS method. After 5 min at 50 °C, the oven temperature was progressively increased to 280 °C over the course of 40 min. The carrier gas was high-purity helium flowing at a rate of 2 mL/min. The 1 µL sample injection was subjected to a split ratio of 1:30, and MS analysis was performed with an ionisation voltage of 70 eV. The phytochemicals were identified by comparing the obtained mass spectra to the NIST library database, a credible source of mass spectral data. Spectral matching was done to verify the compounds, requiring a minimum match percentage of 85 % to guarantee the accuracy and reliability of the identification. [36].

***In silico docking* studies**

Protein preparation

The RCSB Protein Data Bank was used to get the crystal structures of 2 proteins that are important for urolithiasis: Human Adenine Phosphoribosyltransferase (PDB: 1ORE) with a resolution of 2.10 Å and Human Glyoxylate Reductase Hydroxypyruvate Reductase (PDB: 2WWR) with a resolution of 2.82 Å. The BIOVIA software made it easier to get rid of water molecules and heteroatom, and it also added polar hydrogen atoms to the targets and stored the proteins in the 'pdb' format. **Figures 1(A) - 1(B)** presents the prepared protein structures.



(A) (B)

Figure 1 (A): Prepared protein of human adenine phosphoribosyltransferase and (B): Prepared protein of human glyoxylate reductase hydroxypyruvate reductase.

Ligand preparation

In total, 138 compounds were identified in the GC-MS analysis of *Ecbolium viride*, of which 81 were characterized using ADME analysis. Subsequently, 31 compounds were selected for further study through PROTOX analysis (**Tables 1** and **2**). The PubChem database was used to obtain the structures of all phytoconstituents in 'sdf' format. The chemical structures of the 31 phytoconstituents and standard drugs, including Allopurinol, Febuxostat,

Hydrochlorothiazide, Penicillamine, Potassium Citrate, Tamsulosin, and Tiopronin, were converted to 'pdb' format by adding polar groups to the ligands using BIOVIA software.

Table 1 The GC-MS analysis of extract of *Ecbolium viride* leaves.

S. No.	Retention time	Chemical compound	Molecular formula
1	9.455	(S)-Tetrahydrofuran-3-ol	C4H8O2
2	9.039	Tri methyl ammonium acetate hydrochloride	C5H12ClNO2
3	7.778	4-Methyl-1,3-dioxolan-2-1	C4H6O3
4	17.103	3-Ethyl-2,5-dimethyl-1,3-hexadiene	C10H18
5	9.282	1,4-Anhydrohexitol	C6H12O5
6	19.681	(3S,7Z)-3,7,11-Trimethyldodeca-1,6,10-trien-3-ol	C15H26O
7	7.208	3,3-Dimethyl-4-(di methyl amino)-2-phenylbutan-2-ol	C14H23NO
8	27.774	Tetradecan-2-1	C14H28O
9	20.137	3,3,5,5-Tetramethylcyclohexan-1-ol	C10H20O
10	25.003	(Z)-Tetradec-7-enal	C14H26O
11	12.920	Ethyl (4-methoxycyclohexylidene)acetate	C11H18O3
12	8.400	2,7-Dimethyloct-7-en-5-yn-4-yl acetoxyacetate	C14H20O4
13	12.106	2,3-Dihydrobenzofuran	C8H8O
14	16.580	exo-1,2-Dimethylbicyclo[2.2.1]heptan-2-yl acetate	C11H18O2
15	20.421	cis-6-Iodo-2-methylbicyclo[3.3.0]octan-3-1	C9H13IO
16	13.971	Hexyl 3-methylbutanoate	C11H22O2
17	16.726	2,2-Dimethyl-3-(2-methylprop-1-enyl)cyclopropanecarboxylic acid	C19H26O3
18	11.186	Decyl prop-1-en-2-yl carbonate	C14H26O3
19	13.136	Tetradecyl prop-1-en-2-yl carbonate	C18H34O3
20	12.760	Decanoic acid	C10H20O2
21	18.000	Dodecanoic acid	C12H24O2
22	5.336	2-Isopropoxyethanol	C5H12O2
23	7.115	2-Methylhex-3-yl formate	C8H16O2
24	18.229	3-Methylbutyl heptanoate	C12H24O2
25	25.677	Methyl (10E,12Z)-octadeca-10,12-dienoate	C19H34O2
26	7.390	2,6-Dimethylpiperazine	C6H14N2
27	8.772	1,1,3-Triethoxypropane	C9H20O3
28	4.426	1,1-Diethoxy-2-methylpropane	C8H18O2
29	21.076	Tetradecanoic acid	C14H28O2
30	18.742	Undecanal	C11H22O
31	28.164	(5Z,8Z,11Z,14Z,17Z)-Icosa-5,8,11,14,17-pentaenoic acid	C20H30O2

Table 2 Lipinski's rule for chemical compounds of *Ecbolium viride* extract by SwissADME predictor.

S. No.	Compound	Molecular weight (g/mol)	Hydrogen bond donor	Hydrogen bond acceptor	LogP	Molar refractivity
1	(S)-Tetrahydrofuran-3-ol	88.11	1	2	0.17	21.47
2	Tri methyl ammonium acetate hydrochloride	153.61	0	2	−1.34	35.32
3	4-Methyl-1,3-dioxolan-2-1	102.09	0	3	0.37	21.99
4	3-Ethyl-2,5-dimethyl-1,3-hexadiene	138.25	0	0	3.50	49.24
5	1,4-Anhydrohexitol	164.16	4	5	−1.52	34.57
6	(3S,7Z)-3,7,11-Trimethyldodeca-1,6,10-trien-3-ol	222.37	1	1	4.18	74.00
7	3,3-Dimethyl-4-(di methyl amino)-2-phenylbutan-2-ol	221.34	1	2	2.54	68.74
8	Tetradecan-2-1	212.37	0	1	4.62	69.61
9	3,3,5,5-Tetramethylcyclohexan-1-ol	156.27	1	1	2.59	48.71
10	(Z)-Tetradec-7-enal	210.36	0	1	4.37	69.14
11	Ethyl (4-methoxycyclohexylidene)acetate	198.26	0	3	2.02	54.77
12	2,7-Dimethyloct-7-en-5-yn-4-yl acetoxyacetate	252.31	0	4	2.80	69.67
13	2,3-Dihydrobenzofuran	221.25	1	3	2.15	60.13
14	exo-1,2-Dimethylbicyclo[2.2.1]heptan-2-yl acetate	182.26	0	2	2.54	51.83
15	cis-6-Iodo-2-methylbicyclo[3.3.0]octan-3-1	264.10	0	1	2.52	54.31
16	Hexyl 3-methylbutanoate	186.29	0	2	3.21	56.28
17	2,2-Dimethyl-3-(2-methylprop-1-enyl)cyclopropanecarboxylic acid	350.45	0	3	5.24	104.43
18	Decyl prop-1-en-2-yl carbonate	242.35	0	3	4.37	71.70
19	Tetradecyl prop-1-en-2-yl carbonate	242.35	0	3	4.37	71.70
20	Decanoic acid	172.26	1	2	3.00	51.96
21	Dodecanoic acid	200.32	1	2	3.51	61.57
22	2-Isopropoxyethanol	104.15	1	2	0.58	28.40
23	2-Methylhex-3-yl formate	144.21	0	2	2.16	42.24
24	3-Methylbutyl heptanoate	200.32	0	2	3.59	61.08
25	Methyl (10E,12Z)-octadeca-10,12-dienoate	294.47	0	2	5.97	93.78
26	2,6-Dimethylpiperazine	427.54	2	6	2.41	129.51

S. No.	Compound	Molecular weight (g/mol)	Hydrogen bond donor	Hydrogen bond acceptor	LogP	Molar refractivity
27	1,1,3-Triethoxypropane	176.25	0	3	1.83	48.63
28	1,1-Diethoxy-2-methylpropane	146.23	0	2	2.02	42.74
29	Tetradecanoic acid	146.23	0	2	2.02	42.74
30	Undecanal	170.29	0	1	3.55	55.19
31	(5Z,8Z,11Z,14Z,17Z)-Icosa-5,8,11,14,17-pentaenoic acid	302.45	1	2	5.99	97.66

Molecular docking

Molecular docking studies were conducted on all phytoconstituents and standard drugs against the Human Adenine Phosphoribosyltransferase and Human Glyoxylate Reductase Hydroxypyruvate Reductase targets using the PyRx tool of autodockvina software. PyRx software is used to open the prepared protein and ligands, minimize all of the ligands, and convert the protein and ligand into the "PDBQT" format. The docking technique was then conducted after generating the grid parameter configuration file. The standard drugs were compared to the binding energies of all the phytoconstituents that were saved.

Analysis and visualization

Biovia Drug Discovery Studio was used to visualize the 2D and 3D structures of compounds with the highest binding affinities, as well as to validate the amino acid interactions.

ADMET prediction analysis

Important filter criteria for predicting the drug-like properties and also monitoring the safety and effectiveness of the compounds are absorption, distribution, metabolism, excretion, and toxicity studies. With the help of computational data, it may help to lower the failure of compounds in the experimental evaluation, and it is also cost-effective and time-consuming. We looked into physiochemical and pharmacokinetic properties using SWISS ADME and PREADMET software, as well as bioactivity prediction set up by MOLINSPIRATION software. We also looked into toxicity using PROTOX-II software [37].

***In vitro* anti-urolithiasis activity**

Nucleation assay

A buffer solution was made up with 0.05 M Tris-HCl and 0.15 M NaCl at pH 6.5, accompanied by distinct solutions of 5 mM calcium chloride and 7.5 M sodium oxalate. To commence the crystallization process, 9 mL of calcium chloride solution was combined with 1 mL of the extract at varying concentrations (100, 200, 400, 800, and 1600 µg/mL). Subsequently, 950 mL of sodium oxalate solution was included into the mixture. The temperature was sustained at 37 °C during the operation. After 30 min, the optical density of the solution was assessed at 620 nm. The nucleation rate was assessed by analyzing the induction time of samples containing the extract and Cystone, relative to ones devoid of these components [38]. Crystal growth was anticipated to transpire in the subsequent reaction.

$$CaCl2 + Na2C2O4 \rightarrow CaC2O4 + 2NaCl \quad (1)$$

Aggregation assay

The aggregation method was altered and evaluated utilizing a multiple electrode aggregometer, as delineated by Atmani *et al.* [39]. Calcium oxalate crystals were employed at a concentration of 0.8 mg/mL in a buffered solution comprising 0.05 M Tris–HCl and 0.15 M NaCl at pH 6.5. The experiments were conducted at 37 °C, with or without the addition of test or control samples. The aggregation inhibition percentage was determined by comparing the turbidity of test samples at different concentrations (100, 200, 400, 800, and 1600 µg/mL) to that of the control, employing the subsequent formula:

$$\% \text{ inhibition} = \frac{1-(\text{Turbidity Sample})}{\text{Turbidity Control}} 100\ \% \quad (2)$$

Egg membrane assay

Semi-permeable membranes preparation

The apex of each egg was perforated using a glass rod to facilitate the removal of its contents. The empty eggshells were then meticulously washed with distilled water and subsequently placed in a beaker containing a 2 M hydrochloric acid solution for decalcification, which was allowed to proceed overnight. This process effectively dissolved the calcium carbonate, leaving the semi permeable membrane intact. The membranes were carefully extracted, thoroughly rinsed with distilled water, and subjected to neutralization through immersion in an ammonia solution. After neutralization, the membranes were again rinsed with distilled water to eliminate any residual acid. To preserve their structural integrity and prevent dehydration, the membranes were stored under controlled conditions in a refrigerated environment, with the pH maintained within the range of 7 to 7.4.

Estimation of calcium oxalate using dissolution model

Each egg's semi permeable membrane was carefully encased and immersed in a conical flask containing 100 mL of 0.1 M Tris buffer solution, held in place with thread. A stick was positioned at the mouth of the flask, while the opposite end of the thread was secured with aluminum foil to ensure the membrane remained suspended. All conical flasks were incubated at 37 degrees Celsius for duration of 2 h. Following the incubation period, the contents of each membrane were transferred into individual test tubes. Each test tube received 2 mL of 0.5 M sulphuric acid, followed by titration with 0.2 M potassium permanganate (KMnO4) until a faint pink color persisted. The total dissolved calcium oxalate from different concentrations of extracts and standards was determined by subtracting the residual undissolved calcium oxalate from the initial total amount utilised in the experiment. Each millilitre of 0.2 M KMnO4 utilized corresponds to 0.1898 mg of calcium oxalate [40].

Results and discussion

The hydroalcoholic extract of *Ecbolium viride* leaves was extracted by the Soxhlet extraction method, resulting in a total yield of 20.64 %.

Phytochemical screening

The phytochemical analysis of extract of *Ecbolium viride* is shown in the **Table 3**. Alkaloids, flavonoids, tannins, glycosides, carbs, amino acids, and polyphenols are some of the important phytochemicals that can be found in *Ecbolium viride* leaves extract. These findings correspond with prior studies that recognised similar phytochemical studies [41]. These data show that *Ecbolium viride* is a plant that is high in phytochemicals and could be a source of useful bioactive compounds.

Table 3 Phytochemical screening of extract.

S.No	Phytochemical compounds	Results
1	Alkaloids	+
2	Saponins	−
3	Amino acids	+
4	Glycosides	+
5	Steroids	−
6	Flavonoids	+
7	Carbohydrates	+
8	Tannins	+
9	Polyphenols	+
10	Sterols and polyterpenes	−

+: Presence, −: Absence.

GC-MS analysis

The identification of the phytochemical constituents of *Ecbolium viride* was performed by GC–MS analysis. Numerous phytocompounds were tentatively identified using the NIST library. **Figure 2** show chromatogram. The most abundant peak was observed for n-Hexadecanoic acid, The other characteristic peaks were 9,12,15-Octadecatrienoic acid, (Z, Z, Z)-, Phytol, methyl (Z, 12R)-12-acetyloxyoctadec-9-enoate, Neophytadiene.

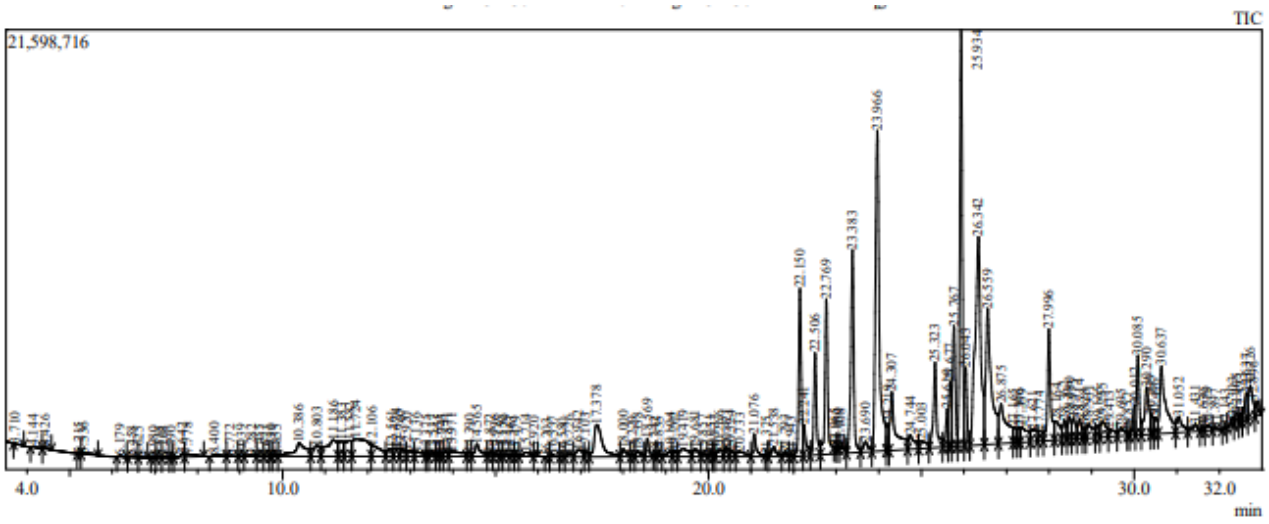


Figure 2 GC chromatogram of *Ecbolium viride* leaves extract.

Docking studies

Molecular docking studies are very helpful in identifying the potential compound exhibiting biological activity through the protein and ligand interactions. Pyrx software is a virtual screening tool used for docking studies. In this study, about 32 phytoconstiutents of *Ecbolium viride* were docked against the 2 targets: Human Adenine Phosphoribosyltransferase (1ORE) and Human Glyoxylate Reductase Hydroxypyruvate Reductase (2WWR) of urolithiasis to identify the lead molecule to combat the kidney stones.

All the ligands exhibit various binding affinities (ΔG kcal/mol) ranging against both the targets. In this Piperazine, 2, 6-dimethyl shows higher affinity of −6.9 kcal/mol in phytoconstituents and Febuxostat shows higher affinity of −6.7 kcal/mol in standard drugs against Human Adenine Phosphoribosyltransferase are shown in **Tables 4** and **5**. Whereas against Human Glyoxylate Reductase Hydroxypyruvate Reductase again piperazine, 2, 6-dimethyl shows higher affinity of −7.6 kcal/mol in phyto constituents and Febuxostat shows higher affinity of −7.3 kcal/mol in standard drugs are shown in **Tables 4** and **5**.

2D & 3D interaction of 2,6-Dimethylpiperazine forms binding interaction with Asp 127 and Febuxostat forms binding interaction with Leu A:162, Ala 131, Val 24 of the active site of the Human Adenine Phosphoribosyltransferase was shown in **Figures 3(A) - 3(B)**. Whereas 2D & 3D interaction of 2,6-Dimethyl piperazine forms binding interaction with Gly D: 84 and Febuxostat forms binding interaction with Leu B:59, His B:293 of the active site of the Human Glyoxylate Reductase/ Hydroxypyruvate Reductase were shown in the **Figures 4(A) - 4(B)**.

Table 4 Docking score of phytocompounds.

S. No.	Phytoconstituents	Binding affinity (1ORE)	Binding affinity (2WWR)
1	(S)-Tetrahydrofuran-3-ol	−3.3	−3.5
2	Tri methyl ammonium acetate hydrochloride	−3.8	−3.5
3	4-Methyl-1,3-dioxolan-2-1	−3.9	−3.5
4	3-Ethyl-2,5-dimethyl-1,3-hexadiene	−4.6	−4.5
5	1,4-Anhydrohexitol	−5	−5.4
6	(3S,7Z)-3,7,11-Trimethyldodeca-1,6,10-trien-3-ol	−5.2	−5.1
7	3,3-Dimethyl-4-(di methyl amino)-2-phenylbutan-2-ol	−5.4	−6.1
8	Tetradecan-2-1	−4.6	−4.8
9	3,3,5,5-Tetramethylcyclohexan-1-ol	−5.1	−5.6
10	(Z)-Tetradec-7-enal	−4.9	−4.6
11	Ethyl (4-methoxycyclohexylidene)acetate	−5.1	−5.2
12	2,7-Dimethyloct-7-en-5-yn-4-yl acetoxyacetate	−1.1	−1.2
13	2,3-Dihydrobenzofuran	−5.9	−5.9
14	exo-1,2-Dimethylbicyclo[2.2.1]heptan-2-yl acetate	−5.1	−5.5
15	cis-6-Iodo-2-methylbicyclo[3.3.0]octan-3-1	−4.9	−5.1
16	Hexyl 3-methylbutanoate	−4.3	−4.7
17	2,2-Dimethyl-3-(2-methylprop-1-enyl)cyclopropanecarboxylic acid	−6.4	−6.4
18	Decyl prop-1-en-2-yl carbonate	−5.1	−4.5
19	Tetradecyl prop-1-en-2-yl carbonate	−5.2	−4.8
20	Decanoic acid	−4.6	−4.8
21	Dodecanoic acid	−4.5	−4.6
22	2-Isopropoxyethanol	−3.3	−3.4
23	2-Methylhex-3-yl formate	−3.8	−4.1
24	3-Methylbutyl heptanoate	−4.2	−4.5
25	Methyl (10E,12Z)-octadeca-10,12-dienoate	−4.7	−5.1
26	2,6-Dimethylpiperazine	**−6.9**	**−7.6**
27	1,1,3-Triethoxypropane	−3.4	−3.5
28	1,1-Diethoxy-2-methylpropane	−3.9	−3.9
29	Tetradecanoic acid	−4.6	−4.8
30	Undecanal	−4.2	−4.3
31	(5Z,8Z,11Z,14Z,17Z)-Icosa-5,8,11,14,17-pentaenoic acid	−5.2	−5

Table 5 Docking score of Standard drugs.

S. No.	Phytoconstituents	Binding affinity (1ORE)	Binding affinity (2WWR)
1	Febuxostat	−6.7	−6
2	Hydrochlorothiazide	−6.5	−7.3
3	Tamsulosin	−6.1	−6.2
4	Allopurinol	−5.5	−4.1
5	Tiopronin	−4.3	−1.7
6	Penicillamine	−4.1	−6.2
7	Potassium Citrate	−1.2	−4.5

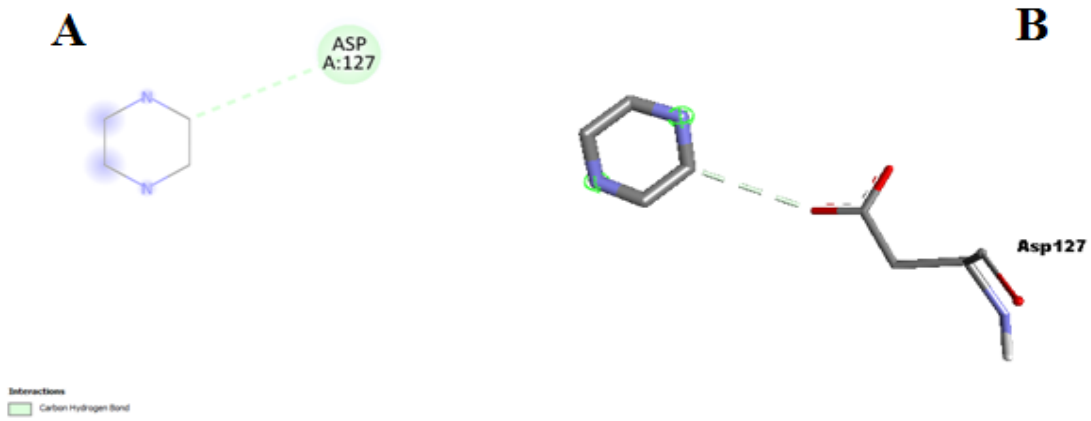


Figure 3 Interactions of 2,6-Dimethylpiperazine with human adenine phosphoribosyltransferase (1ORE); (A) 2D structure and (B) 3D Structure.

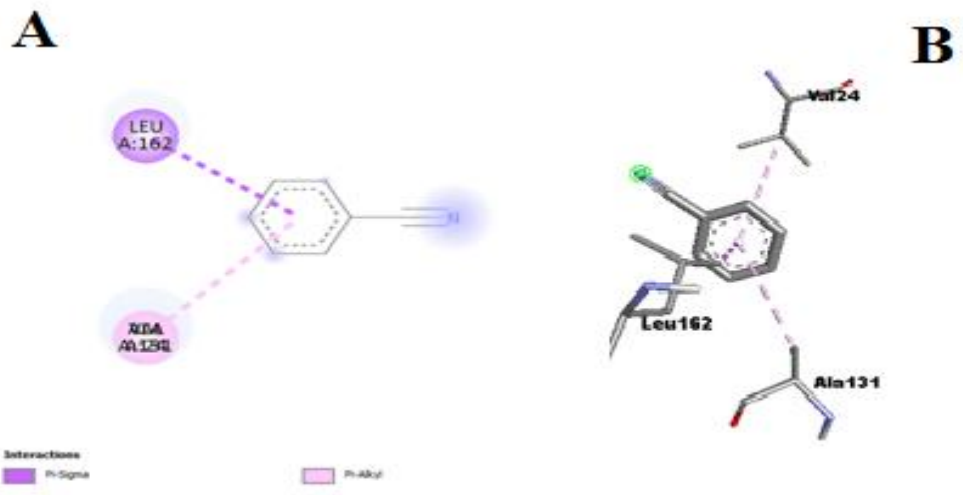


Figure 4 Interactions of febuxostat with human glyoxylate reductase/hydroxypyruvate reductase (2WWR); (A) 2D structure and (B) 3D structure.

***In vitro* anti Urolithiasis activity**

Nucleation assay

The stages of stone production include nucleation, crystallization, and aggregation. As a result, various assays were utilized to evaluate the anti-urolithiatic effectiveness of the combined extract [42]. Cystone is utilized for the treatment of urolithiasis and renal calculi, comprising a combination of several plants, such as *Didymocarpus pedicellata*, *Bergenia ligulata* and Gokshura. Palaniyamma and Jeyaraman employed cystone as a standard [43]. Nucleation denotes a thermodynamically driven phase change wherein solutes in a supersaturated solution crystallize spontaneously. A marked reduction in the nucleation of calcium oxalate (CaOx) crystals was observed when using the extract and Cystone, highlighting the extract's efficacy in inhibiting crystallization in the CaOx crystallization assay. The proposed mechanism underlying the extract's anti crystallization effect likely involves its capacity to chelate free calcium and oxalate ions, thereby impeding the formation of CaOx complexes. The polymorphism of calcium oxalate, a phenomenon of considerable importance, is frequently encountered in urolithiasis and plays a critical role in the formation and growth of renal stones. **Figure 5** illustrate the results of the nucleation inhibition activity of *Ecbolium viridae* extract under *In vitro* conditions. The inhibitory efficacy of *Ecbolium viridae* extract reached 82.3 ± 1.44 % at a dose of 1600 µg/mL. The inhibitory effect has been amplified by elevating the concentration. A statistical investigation using the Student's t-test revealed a significant difference between Cystone and the extract ($p < 0.05$). The extract may be anti-urolithiatic, but less so than Cystone. The results of the nucleation experiment confirmed that the extract included compounds that impede nucleation, hence averting the development of kidney stones.

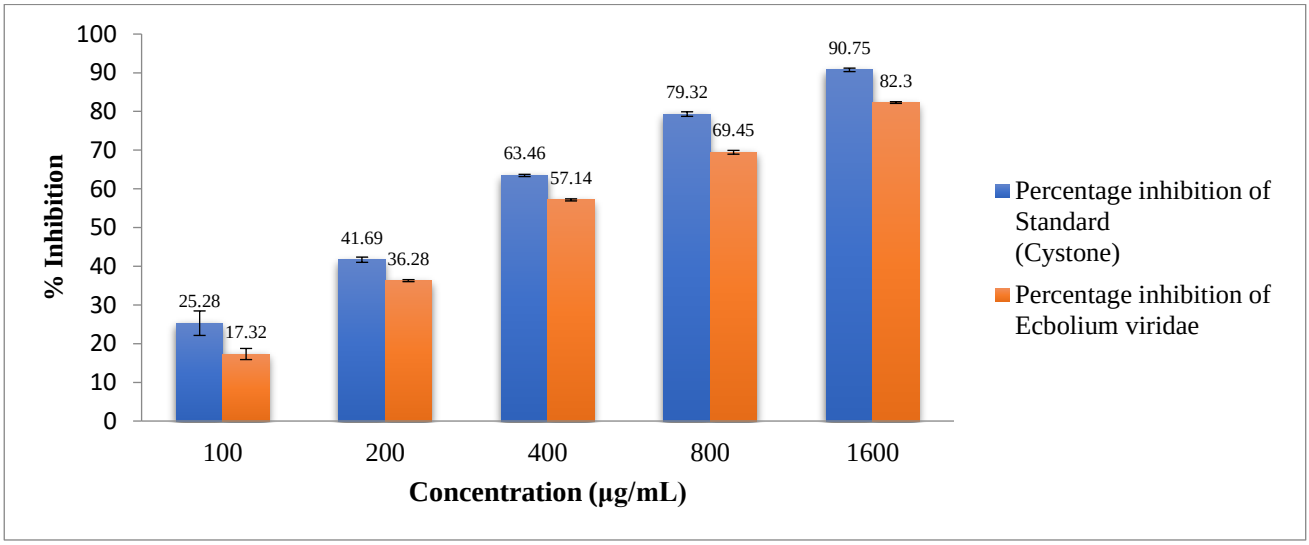


Figure 5 Effect of *Ecbolium viride* and Cystone on nucleation.

Aggregation assay

Aggregation is the second most critical factor in the formation of kidney stones. It is the mechanism that enhances the dimensions, composition, and structure of urinary calculi [45]. The aggregation of crystals refers to the process in which several crystals in a solution coalesce and attach to create enormous crystal clusters. Aggregation is a crucial factor in crystal retention, as substantial crystal agglomerates are responsible for renal tubular blockage, thereby facilitating stone development [44]. **Figure 6** illustrates the capacity of *Ecbolium viridae* extract to dissolve crystals and their effect on calcium oxalate aggregation. While the rate of inhibition elevated with increase in concentration of the extract. Percent inhibition aggregation produced by *Ecbolium viride* was found to be 70.15 ± 0.24 % comparable to that of Cystone (83.79 ± 0.29 %) at 1600 µg/mL concentration. A Student's t-test was used to ascertain the type of relationship between Cystone and *Ecbolium viridae's* inhibitory capacity at each concentration. According to the statistical analysis's findings, the *p*-values show that, at every dose

examined, the variations in the inhibitory efficiency between Cystone and *Ecbolium viridae* are statistically significant ($p < 0.05$). Considering all of this, it can be said that Cystone is far more effective in treating this condition, even if Ecbolium viridae has strong anti-urolithiatic properties.

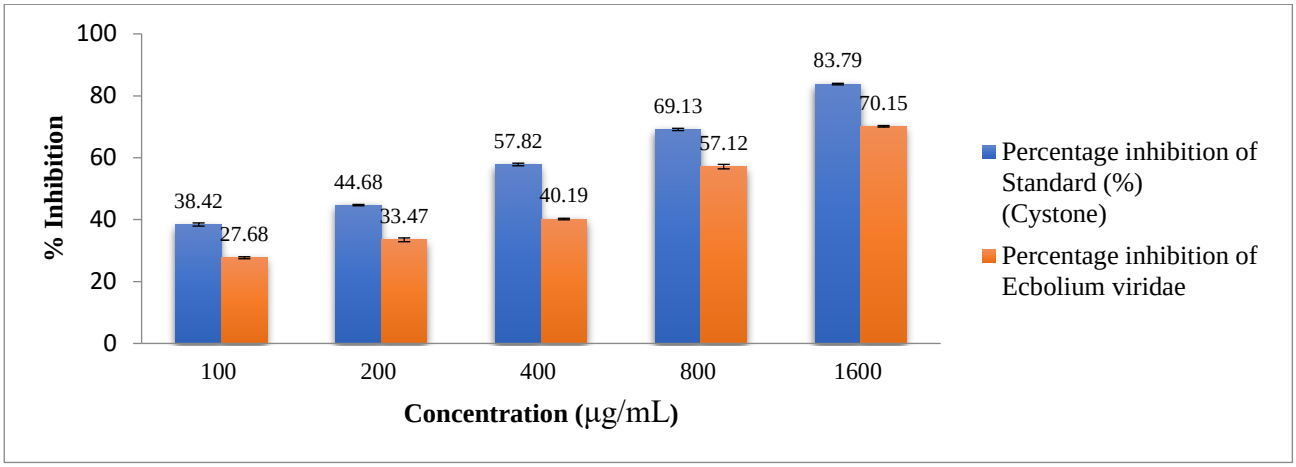


Figure 6 Effect of *Ecbolium viride* and Cystone on aggregation.

Dissolution of calcium oxalate crystals by titrimetry assay

The amount of CaOx dissolved was nominated as the indicator to evaluate anti-urolithiatic activity. The findings from the capacity of plant extract to dissolve CaOx crystals can be achieved effectively with a minimal quantity, in contrast to its inhibitory activity [46]. The antiurolithiatic activity of *Ecbolium viride* aligns with the reported efficacy of other Acanthaceae plants [25]. The dissolution percentage results of CaOx using plant extracts and the standard are presented in **Table 6**. The dissolution of CaOx with the standard drug was measured at 48.4 ± 0.16 %, representing the highest percentage of dissolution in comparison to the plant extracts. Extract concentrations ranged from 67.22 ± 1.24 % to 46.37 ± 0.57 %, indicating their ability to dissolve CaOx. A lower percentage indicates a more effective breakdown of calcium oxalate crystals. The extract at a concentration of 500 mg/mL has a superior capacity to dissolve calcium oxalate and exhibits demineralization comparable to that of the standard medication Cystone. It's demonstrated that *Ecbolium viride* exhibits significant anti-urolithiatic activity by effectively dissolving CaOx crystals, even at minimal concentrations of phytochemicals. The *p*-values suggest that the difference in dissolution percentages between Cystone and Ecbolium viridae extract is statistically significant ($p < 0.05$) at all concentrations that were measured.

Table 6 The percentage of dissolution on CaOx crystals by plant extract of *Ecbolium viride* and standard drugs (cystone).

S.No	Concentration (mg/mL)	Standard drug (Cystone) dissolution percentage (%)	Test drug (*Ecbolium viridae* extract) Dissolution percentage (%)
1	100	63.5 ± 0.40	67.22 ± 1.24
2	200	60.5 ± 0.21	64.22 ± 1.24
3	300	58.7 ± 0.15	60.54 ± 0.76
4	400	52.38 ± 0.26	54.77 ± 0.34
5	500	48.4 ± 0.16	46.37 ± 0.57

Conclusions

This research indicates that *Ecbolium viride* may serve as a natural remedy for urolithiasis. This is supported by evidence from phytochemical, computational, and *in vitro* research. *In vitro* experiments, including nucleation, aggregation, and dissolution of calcium oxalate crystals, confirmed that *Ecbolium viride* extract significantly prevented calcium oxalate crystal growth and aggregation while favoring their dissolution. Further *in vivo* and clinical trials investigation is required prior to the potential approval of *ecbolium viride* as a phytotherapeutic treatment for urolithiasis.

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