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PHARMACOGNOSTIC PHYTOCHEMICAL STANDARDIZATION AND INVITRO TESTING OF ANTI_UROLITHIATIC AND NEPHROPROTECTIVE ACTIVITY IN EXCOECARIA AGALLOCHA LEAVES

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ABSTRACT
The Pharmacognostic, phytochemical and pharmacological studies of the leaves of Excoecaria agallocha L. Pharmacognostical Studies, Phytochemical screening of the extract, Identification of quercetin in hydroalcoholic extract of Excoecaria agallocha leaves (HAEEA) by HPTLC method. In-silico Molecular docking studies to check the effect of anti-oxidant potential of the extract by using DPPH (2-Diphenyl 1-2 picrylhydrazyl) assay and Nitric Oxide Scavenging Assay. anti-urolithiatic activity of hydroalcoholic extract of Excoecaria agallocha (HAEEA) by nucleation assay, nephroprotective activity of hydroalcoholic extract of Excoecaria agallocha (HAEEA) against cisplatin induced nephrotoxicity in In-vitro Vero cell lines. Studies on the morphology and micromorphology of medicinal plants have always been accorded due credence in pharmacognosy. It is essential to identify the botanical identity of the plants before analyzing their medicinal properties. There is a possibility that a researcher can discover a new compound in a plant or that the plant has many useful pharmacological properties. Hydroalcoholic extract of Excoecaria agallocha leaves was very effective in the management of urolithiasis and cisplatin induced nephrotoxicity, which may be due to the presence of quercetin. The presence of quercetin in HAEEA is likely responsible for these effects, as quercetin has previously been reported to play a crucial role in kidney function. Utilizing computer aided drug design, quercetin could serve as a lead molecule for developing new synthetic drugs to treat kidney-diseased patients.

KEYWORDS: Excoecaria Agallocha, Anti-Urolithiatic, Nephroprotective.

INTRODUCTION
Medicinal plants have been integral to human health and medicine throughout history, serving as the foundation for many traditional and modern medical practices. Their importance is particularly pronounced in systems like Ayurveda, Siddha, and Unani, which predominantly utilize plant-based materials for therapeutic purposes. The use of medicinal plants dates back to prehistoric times, with evidence suggesting that early humans relied on local flora for healing. Ancient texts, such as the Rig-Veda from India (circa 1600-3500 B.C.), document the medicinal properties of various plants. Similarly, the Ebers Papyrus from ancient Egypt (circa 1550 B.C.) lists over 850 plant-based remedies, illustrating the long-standing relationship between humans and medicinal herbs. According to the World Health Organization (WHO), approximately 80% of the population in developing countries relies on traditional medicine, much of which is derived from medicinal plants. This reliance highlights the essential role that these plants play in healthcare systems where access to modern pharmaceuticals may be limited. Moreover, many contemporary medications are derived from compounds found in these plants; for instance, drugs for childhood cancers have origins in traditional herbal remedies like the Madagascar periwinkle.[1-5]

Medicinal plants are celebrated not only for their historical significance but also for their diverse therapeutic properties. They contain a variety of bioactive compounds that can exhibit anti-inflammatory, antibacterial, and antifungal effects.[6-8]

Despite their benefits, the sustainable management of medicinal plants faces challenges due to overharvesting and habitat loss. Globalization has increased demand for these resources, often leading to exploitative practices that threaten their availability. Therefore, there is a pressing need for conservation efforts that incorporate traditional knowledge to ensure the sustainability of

these vital resources. Medicinal plants remain a cornerstone of healthcare across cultures. Their historical use informs current practices and highlights an ongoing need to balance utilization with conservation to maintain their availability for future generations.[9-12]

Excoecaria agallocha, commonly known as the milky mangrove or blinding tree, is a species of mangrove found primarily in tropical and subtropical regions. Its leaves exhibit distinct morphological characteristics and have significant ecological and medicinal implications. Excoecaria agallocha plays a crucial role in coastal ecosystems by stabilizing shorelines and providing habitat for various marine species. It is typically found in back mangrove areas where salinity is lower than that of more coastal mangrove species.[13-16]

Urolithiasis, commonly referred to as kidney stone disease, is characterized by the formation of solid mineral deposits (renal calculi) within the urinary tract. These stones typically originate in the kidneys and can cause significant pain and complications if they obstruct the urinary flow. Urolithiasis is prevalent, affecting approximately 1 in 11 individuals in the United States, leading to about 1 million emergency department visits annually. The condition is more common in men than women, with a prevalence of 10.6% versus 7.1%, respectively. Factors such as obesity and certain ethnic backgrounds also influence stone formation rates, with non-Hispanic white males being particularly affected.[17-19]

MATERIALS AND METHODS

PLANT COLLECTION AND AUTHENTICATION: Leaves of Excoecaria agallocha Linn were collected from local area of Namakkal District and were authenticated by Dr. K.N. Sunil Kumar, Research Officer, Siddha central Research Institute, Chennai. The herbarium of this specimen was kept in the department for future reference.

PHARMACOGNOSTICAL STUDIES: Studies on the morphology and micromorphology of medicinal plants have always been accorded due credence in pharmacognosy. It is essential to identify the botanical identity of the plants before analyzing their medicinal properties. There is a possibility that a researcher can discover a new compound in a plant or that the plant has many useful pharmacological properties.[20-21]

MACROSCOPICAL STUDIES: A variety of organoleptic properties were evaluated, including the shape, size, colour, odour, taste, and fracture of a stem bark, the margin and apex of a leaf, its base surface, the venation, and the inflorescence.[22]

MICROSCOPICAL STUDIES[23-35]

Transverse section of Excoecaria agallocha leaves

- Collection of Specimen
- Dehydration
- Infiltration with Paraffin Wax
- Casting to Mold
- Sectioning
- Photo Micrographs
- Quantitative Microscopy of Excoecaria agallocha L
- Pharmacological Studies

RESULTS

Pharmacognostical studies: The Pharmacognostical parameters were used to find out the adulteration and also ensure the plant identity. The evaluation and standardization of medicinal plants will be helpful to prevent the adulteration. Such studies will also be useful in authentication of the plants and ensures reproducible quality of herbal products which will lead to safety and efficacy of natural products.

Macroscopical studies of E.agallocha Linn: The morphological feature of herbal drugs is so important. This will describe the external characters of the plant material. The Organoleptic features such as shape, size, colour, odour, taste of plant material was evaluated.

Table 1: Macroscopy of Excoecaria agallocha leaves.

S. No	Parameters	Observation
1.	Colour Dorsal Ventral	 Green Light Green
2.	Odour	Aromatic odour
3.	Taste	Bitter taste
4.	Leaf type	Simple
5.	Shape	Elliptic
6.	Apex	Acuminate
7.	Stipulus	Minute
8.	Base	Narrow
9.	Petiole length	2-3 cm
10.	Margin	Entire

Microscopical studies: Microscopical characters of the leaves and stems of this plant were studied and the results were discussed below.

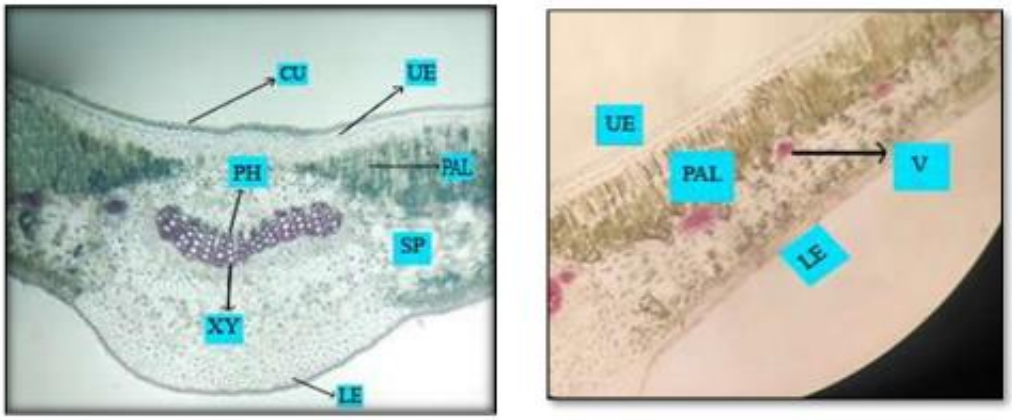


Figure 1: TS of leaf of Excoecaria agallocha (A-midrib portion enlarged; B- Lamina portion enlarged)

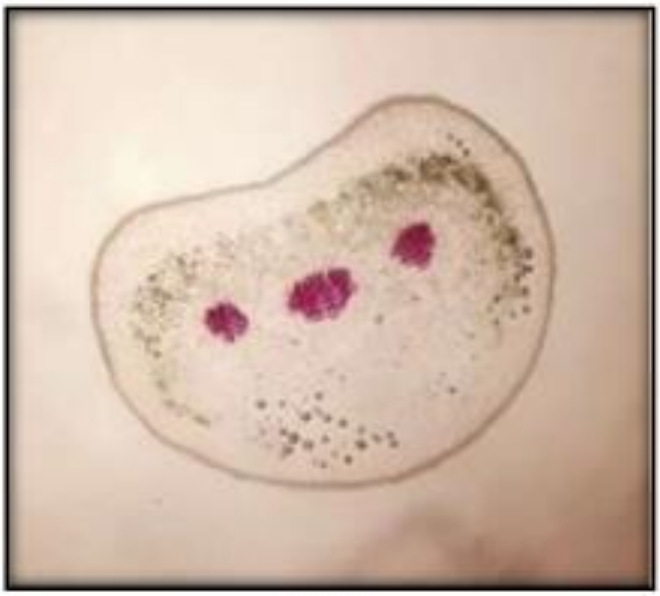


Figure 2: TS of petiole of Excoecaria agallocha L.

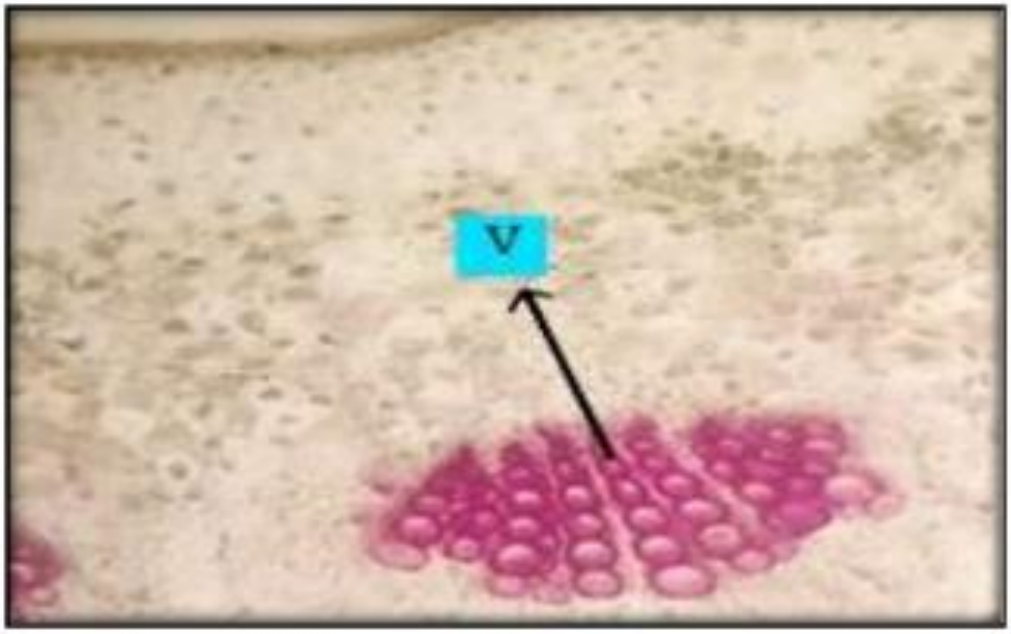


Figure 3: TS of petiole of Excoecaria agallocha (vascular strands portion enlarged).

Quantitative Microscopy

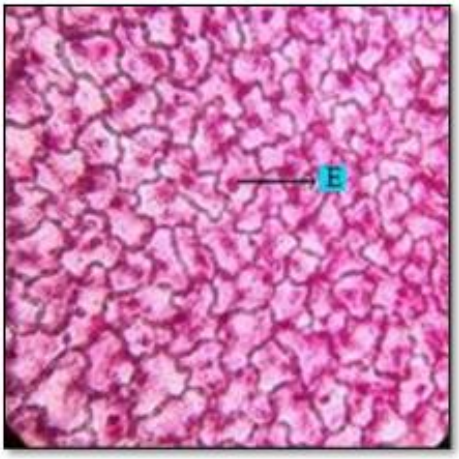


Figure 4: Lower epidermis (10x)

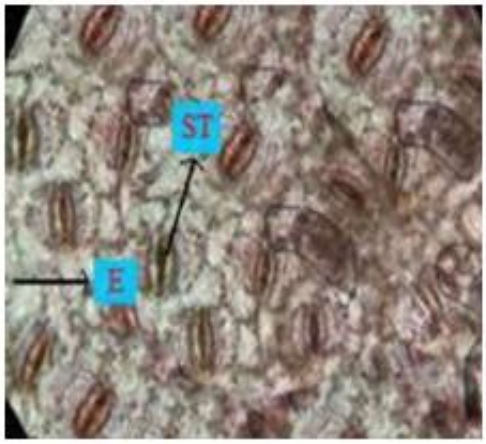


Figure 5: Lower epidermis (45x)

Table 2: Quantitative microscopy Excoecaria agallocha.

S.No	Leaf constant	Observation	
		Upper	Lower
1.	Stomatal index	-	11-18
2.	Stomatal number	-	65-75
3.	Epidermal number	185-235	225-275
4.	Vein islet number	23-28	
5.	Vein termination number	11-16	
6.	Palisade ratio	3:4-3:12	

The upper epidermis was found to have a numbered epidermis of 181-235 and the lower epidermis to have a numbered epidermis of 225-275. The lower epidermis exhibited a Stomatal Index of 11-18. There were 23-28 vein islets, 11-16 vein terminations, and a palisade ratio of 3:4-3:12, with 23-28 vein islets and 11-16 vein terminations.

Powder microscopy

The powdered drugs were evaluated for its physicochemical parameters such as loss on drying, foreign matter, bitterness value, ash value and extractive value. The above results were given that the foreign organic matter of the crude drug material was found to be nil, the percentage of loss on drying was found to be 4.8±0.310%w/w, the percentage of extractive value such as petroleum ether, ethyl acetate, chloroform, ethanol, aqueous was found to be 1.049±0.219%w/w, 0.879±0.48%w/w, 1.417±0.229%w/w, 2.689±0.411%w/w, 1.899±0.38%w/w respectively.

Table 3: Physico-chemical parameters of E. agallocha.

S.No	Physico-Chemical Constant	Observation
1.	Foreign organic matter	Nil
2.	Loss on drying	4.8±0.310%w/w
3.	**Various extractive values**	
	Pet ether	1.049±0.219%w/w
	Ethyl acetate	0.879±0.48%w/w
	Chloroform	1.417±0.229%w/w
	Ethanol	2.689±0.411%w/w
	Aqueous	1.899±0.38%w/w
4.	**Ash value**	
	Total ash	17.24±0.119%w/w
	Acid insoluble ash	02.60±0.138%w/w
	Water soluble ash	12.75±0.412%w/w

Phyto-chemical Analysis

There were a wide range of phytochemicals in the hydro-alcoholic extract (70%) of Excoecaria agallocha (HAEEA); these included alkaloids, carbohydrate, proteins, flavonoids, sterols, triterpenoids, glycosides, tannins, saponins, volatile oils, and mucilage.

An assessment of phytoconstituents quantitatively

Table 4: Estimation of total Phenolic content in HAEEA.

S. No	Concentration (Gallic acid and HAEEA) µg/ml	Absorbance (Mean±SEM)	
		Gallic acid	HAEEA
1.	10	0.057±0.007	0.044±0.001
2.	20	0.109±0.007	0.075±0.002
3.	30	0.142±0.0018	0.102±0.005
4.	40	0.182±0.0019	0.131±0.004
5.	50	0.224±0.0019	0.179±0.001
		GAE	97.72 µg/g

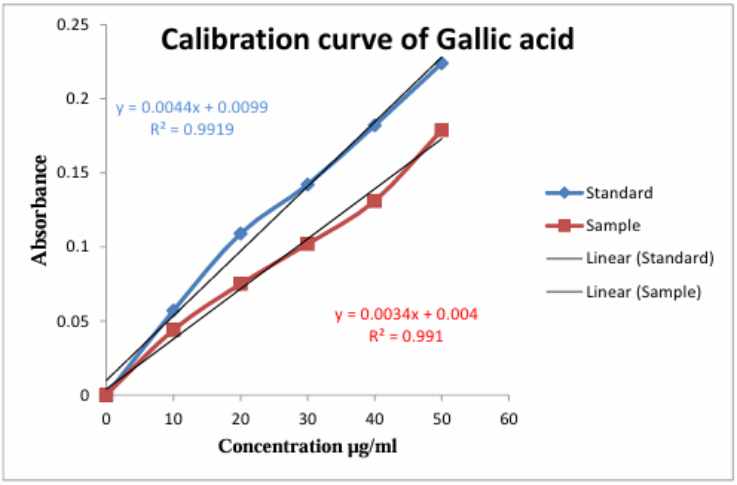


Figure 6: Calibration curve of Gallic acid.

This study revealed that phenolic content in this extract was found to be 97.72 µg/gm.

Table 5: Total Flavonoid content estimation.

S.No	Concentration (Gallic acid and HAEEA) µg/ml	Absorbance (Mean±SEM)	
		Quercetin	HAEEA
1.	10	0.011±0.001	0.003±0.0001
2.	20	0.022±0.003	0.009±0.0001
3.	30	0.034±0.002	0.015±0.0003
4.	40	0.044±0.001	0.021±0.001
5.	50	0.053±0.002	0.028±0.0004
		QE	95.45µg/g

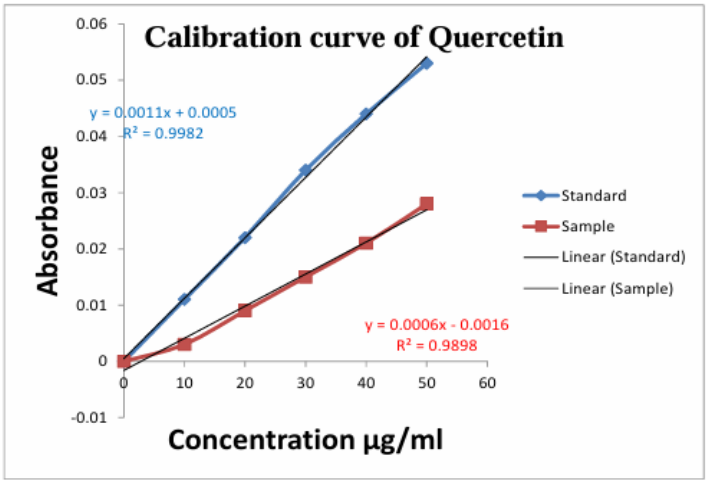


Figure 7: Calibration curve of Quercetin.

This study revealed that flavonoid content in this extract was found to be 95.45µg/gm.

Table 6: Tannin content estimation.

S. No	Concentration (Tannic acid and HAEEA) µg/ml	Absorbance (Mean ± SEM)	
		Tannic acid	HAEEA
1.	10	0.024±0.0002	0.010±0.005
2.	20	0.071±0.00041	0.052±0.004
3.	30	0.111±0.00091	0.088±0.007
4.	40	0.149±0.00052	0.130±0.008
5.	50	0.203±0.00081	0.169±0.004
		TAE	103.46µg/g

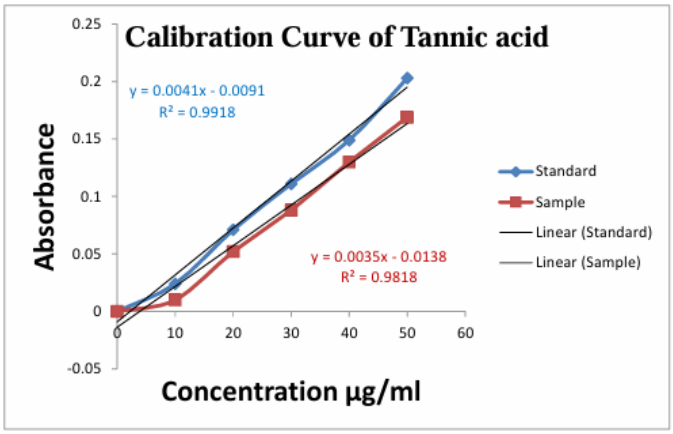


Figure 8: Calibration curve of Tannic acid.

This study revealed that total tannin content in this extract was found to be 103.46µg/gm.

CHROMATOGRAPHIC STUDY

HPTLC Study of HAEEA

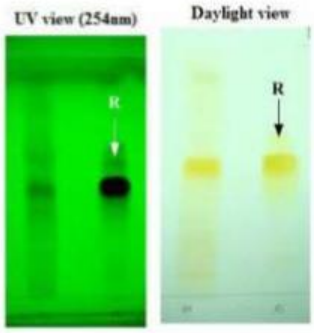


Figure 9: HPTLC Chromatogram of HAEEA.

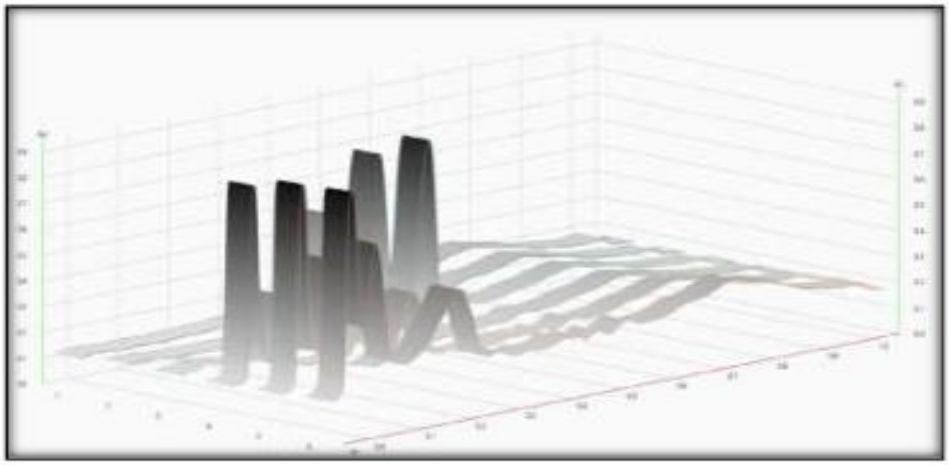


Figure 10: 3D- Display of Standard and HAEEA at 254 nm.

Table 7: HPTLC Profile of HAEEA extract and standard Quercetin.

S. No	Peak Number	Rr Value of sample	Rr Value of Quercetin	UV range
1.	Peak I	0.243		254nm
2.	Peak II	0.397		
3.	Peak III	0.495	0.496	
4.	Peak IV	0.501		
5.	Peak V	0.901		

Chromatogram was developed by HPTLC for hydroalcoholic extract of Excoecaria agallocha (HAEEA) and it showed significant separation of five phytoconstituents whose Rf values ranges from 0.243 to 0.901. Based on HPTLC studies, it was inferred that quercetin was present in the plant extract. It was found that Rf values of a phytoconstituents (quercetin) present in HAEEA almost coincided with the Rf value of standard Quercetin (0.496).

***Insilico* Molecular Docking**

Table 8: Docking scores of ligands with 6FD4.

Compound	CID	docking score	glide emodel
Quercetin	5280343	-8.09	-75.774
Mannitol	6251	-6.952	-68.627
Triterpenoids	71597391	-4.915	-49.461
2',4,4',6'-Tetramethoxychalcone	14034812	-4.342	-55.216
Cycloartenol	92110	-2.954	-28.756
beta-Amyrone	12306160	-2.241	-32.298
beta-Amyrin	73145	-2.207	-30.088
3-Epi-taraxerol	12443227	-2.148	-29.95

Table 9: Docking scores of ligands with protein 6FD4.

Compound	Prime MM/GBSA Binding energy	MM/GBSA Binding H-bond energy	MM/GBSA Binding Van der Waal energy
Quercetin	-4.07	-94.69	-26.90
Mannitol	-3.94	-94.56	2.41
Triterpenoids	-1.31	-91.93	-29.97
2',4,4',6'-Tetramethoxychalcone	-1.84	-92.46	-29.02
Cycloartenol	-0.70	-91.32	-35.41
beta-Amyrone	-0.69	-91.31	-26.37
beta-Amyrin	-0.04	-90.66	-32.75
3-Epi-taraxerol	-0.24	-90.86	-21.30

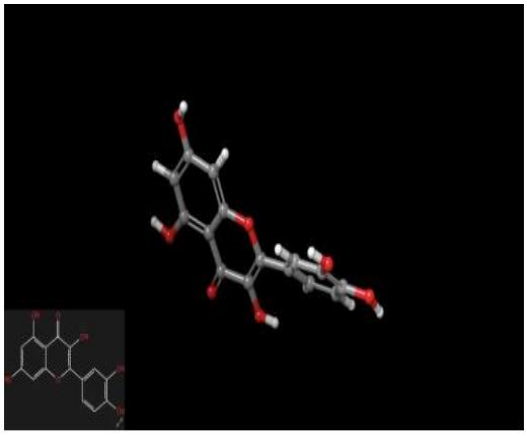


Figure 11: Protein image 6FD4 binding with quercetin.

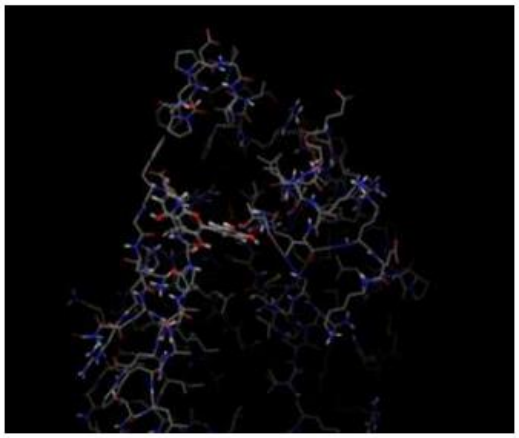


Figure 12: 3D Image of quercetin.

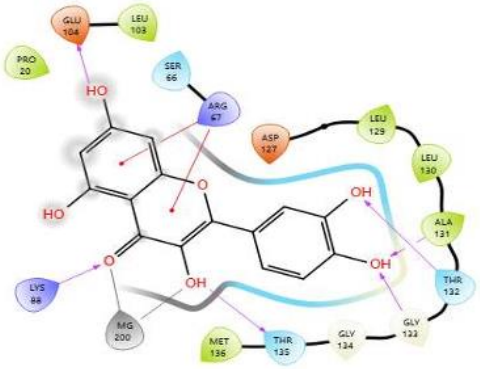


Figure 13: 2D interaction of quercetin with 6FD4 protein.

Molecular Docking: Comparing to other secondary metabolites present in Excoecaria Agallocha, Quercetin had the highest binding score of -8.09.

MM/GBSA: Binding H-bond energy is the energy required for the formation or breaking of hydrogen bond. Similar to binding covalent energy, a negative value indicates a favourable interaction between the ligand and the protein. Here quercetin has highest value of -94.68 when binding with 6FD4. Binding Vander Waal energy indicates interaction between the ligand and protein based on the fluctuation electron density. A positive value indicates that the repulsive forces between the ligand and protein are stronger whereas a negative value indicates that the attractive forces between the ligand and the protein are stronger than the repulsive forces. Here quercetin has-26.90 kcal/mol for 6FD4, showing its attractive nature. Furthermore, the presence of H bond interaction shows that have good binding affinity for quercetin it is-94.69.

Anti-Oxidant studies

Effect of Anti-Oxidant Activity using DPPH (2,2-Diphenyl 1-2 Picryl hydrazyl) Free Radical Scavenging Assay

1. OD Value at 517nm: Control mean OD value: 1.219

Table 10: OD Value of standard and HAEEA.

S. No	Tested Sample concentration µg/ml	OD value at 517 nm in triplicates			Average MEAN ±SEM
1.	Control	1.215	1.225	1.219	1.2196±0.0029
2.	500 µg/ml	0.112	0.117	0.118	0.1156±0.0018
3.	250 µg/ml	0.132	0.131	0.141	0.1346±0.0031
4.	100 µg/ml	0.215	0.229	0.234	0.226±0.0056
5.	50 µg/ml	0.261	0.280	0.270	0.2703±0.0054
6.	10 µg/ml	0.312	0.337	0.386	0.345±0.021
7.	Ascorbic acid	0.06	0.053	0.069	0.0606±0.0046

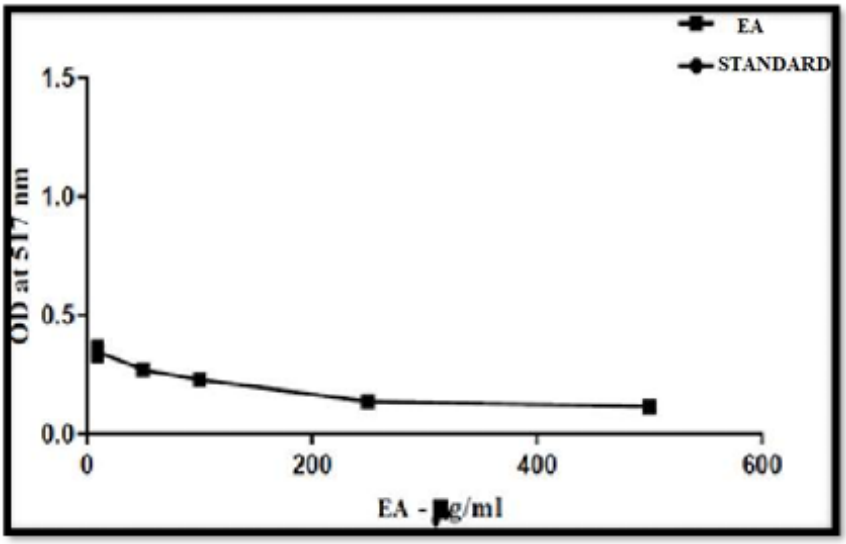


Figure 14: Graphical Representation of in vitro antioxidant potential of HAEEA.

2. Inhibition percentage determination: The IC50 value of HAEEA against DPPH was found to be 27.32 µg/ml and the percentage of inhibition standard ascorbic acid was 96.084 % to that of HAEEA is 82.257%. The percentage of inhibition was found to be in a dose dependent manner.

Table 11: Percentage inhibition of Standard and HAEEA.

S. No	Tested Sample Concentration	% inhibitory absorbance	IC_{50}
1.	Ascorbic acid (1mg/ml)	95.32±0.594	64.38
2.	500 µg/ml	91.73±0.239	27.32
3.	250 µg/ml	90.06±0.229	
4.	100 µg/ml	83.37±0.587	
5.	50 µg/ml	78.37±0.623	
6.	10 µg/ml	73.49±0.441	

Effect of Anti-Oxidant Activity using nitric oxide scavenging activity Free Radical Scavenging Assay

Table 12: Anti-Oxidant Activity using nitric oxide scavenging activity.

Extract (µg/ml)	% of inhibition	
	Hydro-alcoholic extract	Ascorbic acid
100	39.49± 0.036	49.25±0.019
200	51.13 ± 0.025	56.83±0.049
400	60.05± 0.055	65.29±0.039
800	67.23 ± 0.043	69.03±0.073
	IC50 =126.6µg/ml	IC50 = 101.52µg/ml

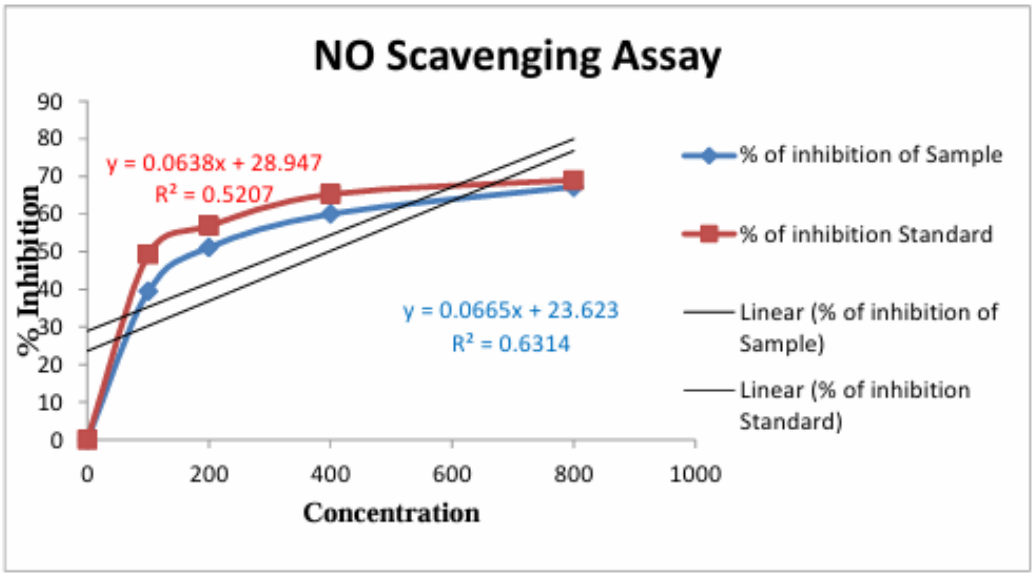


Figure 15: NO Scavenging Assay.

Pharmacological studies

Evaluation of Anti-Urolithiatic activity of the hydro-alcoholic extract of Excoecaria agallocha leaves by nucleation assay

A. OD Value at 620nm

Table 13: OD value of standard and HAEEA (Control mean OD value = 1.235)

S. No	Tested Sample Concentration	OD Value at 620 nm			Average MEAN ±SEM	
1.	Control	1.227	1.238	1.240	1.235	0.004
2.	500 µg/ml	0.631	0.691	0.679	0.667	0.018
3.	250 µg/ml	0.710	0.756	0753	0.739	0.014
4.	100 µg/ml	0.757	0.778	0.824	0.786	0.019
5.	50 µg/ml	0.962	0.947	1.037	0.982	0.027
6.	10 µg/ml	1.286	1.275	1.236	1.265	0.015
7.	Cystone (10mg/ml)	0.335	0302	0.332	0.323	0.010

B. Percentage Inhibition

Table 14: Percentage inhibition of standard and HAEEA.

S.No	Tested Sample Concentration	Mean ± SEM	IC50
1.	Cystone (10mg/ml)	74.48±1.056	38.7
2.	500 µg/ml	46.23±2.042	107.25
3.	250 µg/ml	37.90±2.36	
4.	100 µg/ml	37.38±0.638	
5.	50 µg/ml	23.31±0.716	
6.	10 µg/ml	0±0.000	

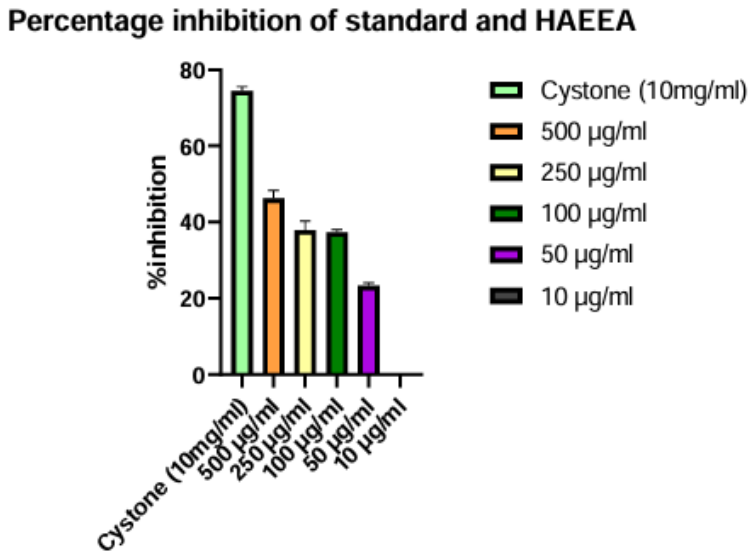
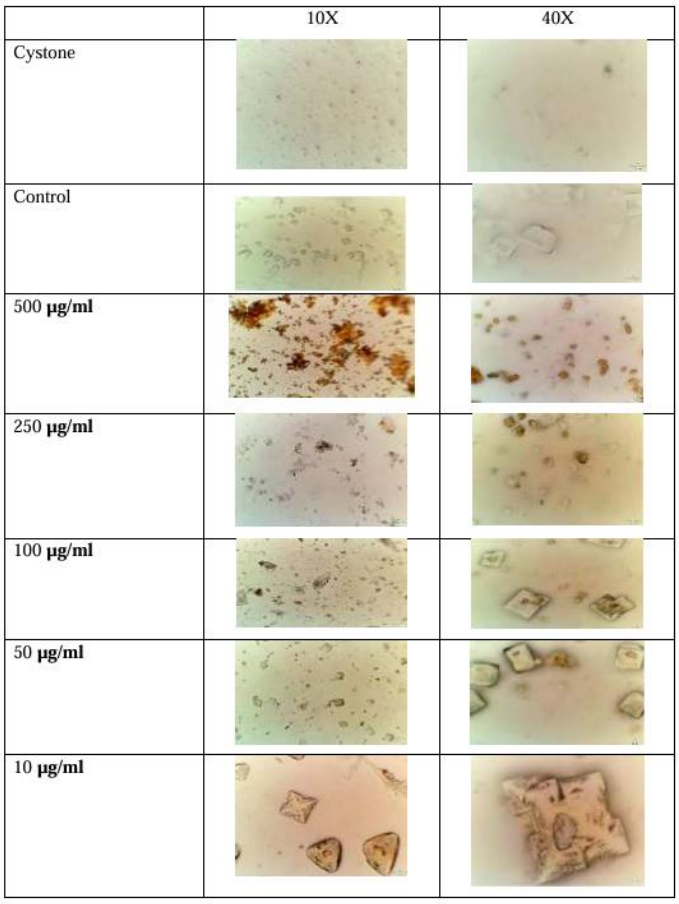


Figure16: Percentage inhibition of standard and HAEEA.



Evaluation of Nephroprotective activity of Hydro-alcoholic extract of Excoecaria agallocha leaves against Cisplatin induced nephrotoxicity in In-vitro Cell line

The cell viability of HAEEA on the VERO cell line was found to be 27.60 mg/ml. Treatment with the (negative control) cisplatin resulted in 40.14% of cell viability accompanied by morphological changes such as cell shrinkage and decreased cell shape. However, on concurrent treatment of cisplatin with various concentrations of HAEEA was found to be 60.11 % of cell viability. This indicates significant improvement of cell viability on HAEEA treatment.

A. Percentage cell viability of HAEEA on VERO cell line

Table 17: Percentage cell viability of HAEEA.

Groups	% Cell viability
Control (15% DMSO)	100±1.18
Test (10 µg/ml)	98.06±2.74
Test (20 µg/ml)	97.35±2.35
Test (50 µg/ml)	95.35±3.31
Test (100 µg/ml)	95.55±3.10
Test (200 µg/ml)	93.44±1.7
IC50 - µg/ml	27.60 µg/ml

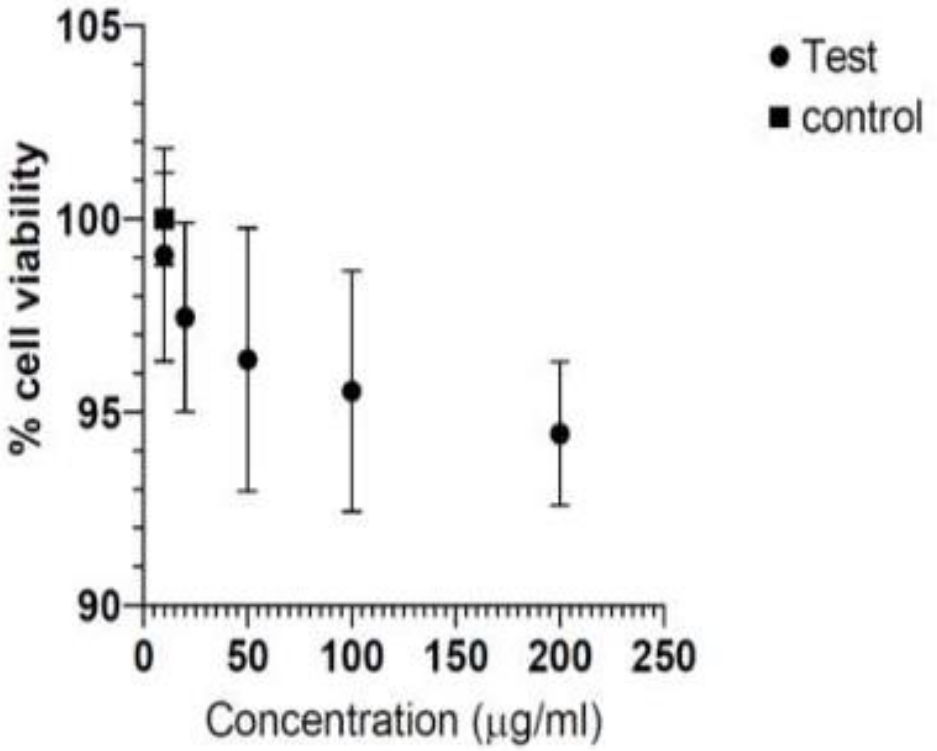


Figure 17: Percentage cell viability of HAEEA on VERO cell line.

B. In-vitro Nephroprotective activity of Hydro-alcoholic extract of Excoecaria agallocha leaves against Cisplatin induced nephrotoxicity

Table 18: Effect of HAEEA on cell viability against Cisplatin induced nephrotoxicity.

Groups	% cell viability
Control (15% DMSO)	100 ± 1.093
Cisplatin (20µM)	40.14 ± 1.45
Cisplatin+ Test (10µg/ml)	46.61± 1.12
Cisplatin+ Test (20µg/ml)	52.10 ± 1.41
Cisplatin+ Test (50µg/ml)	54.41 ± 1.31
Cisplatin+ Test (100µg/ml)	58.40 ± 2.22
Cisplatin+ Test (200µg/ml)	60.11 ± 2.31
EC50	**20.84µg/ml**

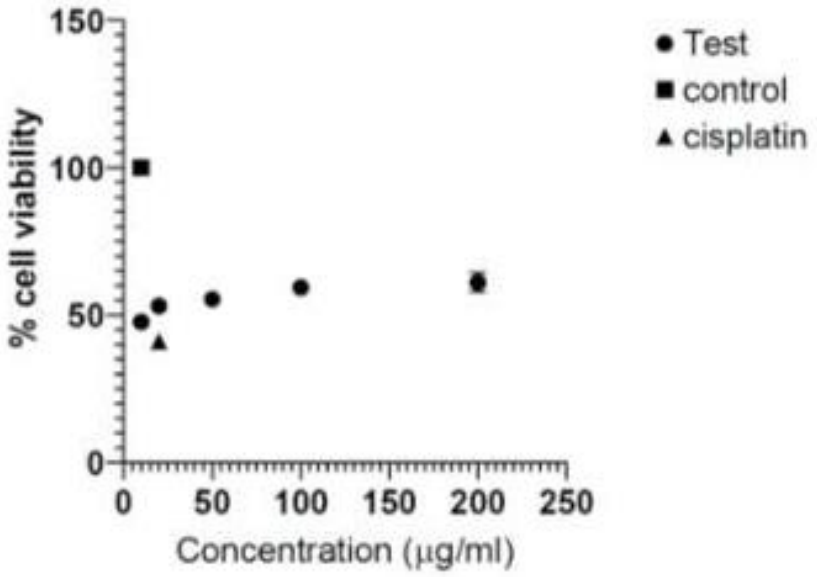


Figure 18: Effect of cell viability against cisplatin induced nephrotoxicity in HAEEA.

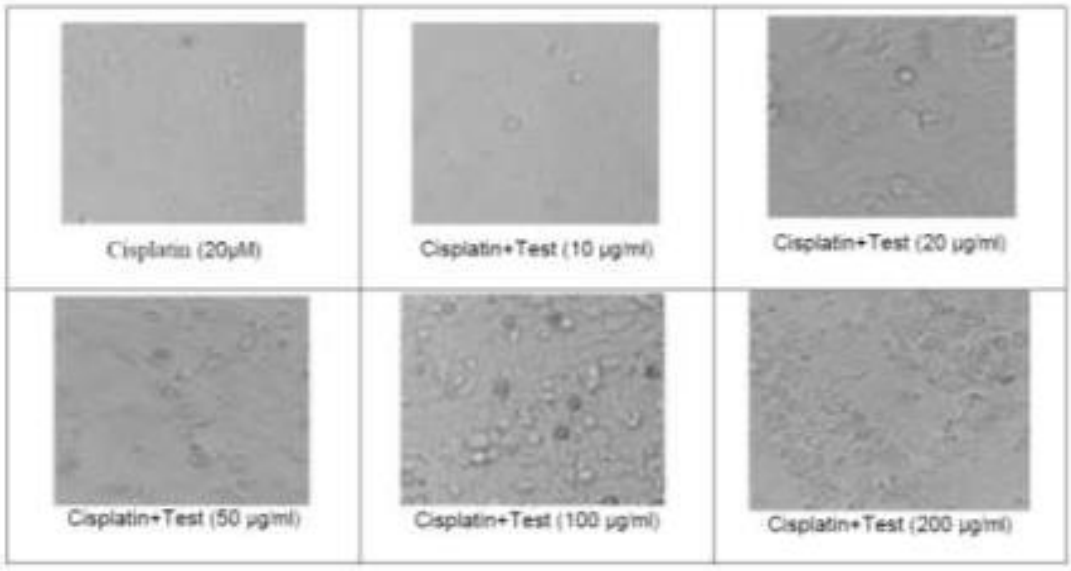


Figure 19: Image analysis of effect of cell viability against Cisplatin induced nephrotoxicity in HAEEA.

DISCUSSION

Cisplatin-induced nephrotoxicity is closely associated with direct DNA damage and mitochondrial dysfunction. This plant plays an important rolein nephron protection by inhibition of inflammatory responses, enhancement of the anti-oxidant effect by reducing mitochondrial DNA damage and reduces the apoptosis of non tumor cells caused by Cisplatin treatment. The compound exhibited, decrease the production of reactive oxygen species (ROS) in the kidneys. ROS are harmful molecules that can damage cells and tissues when present in excessive amount. By reducing ROS production, it enhances anti-oxidant defences and mitochondrial activity and prevents kidney from damage. This is the first time we have reported the nephron-protective potential of HAEEA by cisplatin induced nephrotoxicity in in vitro Vero cell line. Hence it is suggested that HAEEA may be used to treat kidney patients and it may also be given to the cancer patients receiving cancer chemotherapy.

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