Journal of Young Pharmacists, 2025; 17(2):470-476.
https://www.jyoungpharm.org

Original Article

In vitro Anti-Urolithiatic Evaluation of a Polyherbal Formulation against Calcium Oxalate-Induced Urolithiasis in Rats

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ABSTRACT

Background: Kidney stones form due to the crystallization of minerals and salts in the urinary tract. Despite advancements in understanding their pathophysiology, treatment options remain expensive and often lead to recurrence. Herbal therapies have gained attention as alternative treatments. This study evaluates the anti-urolithiatic activity of methanolic extracts of *Nelumbo nucifera, Anogeissus latifolia, Leucas aspera*, and their polyherbal formulation (PHF) in a urolithic rat model. **Materials and Methods:** A 28-day study used a CaOx-induced rat model (70 mg/kg) to induce urolithiasis. Rats received *N. nucifera, A. latifolia*, and *L. aspera* extracts (250/500 mg/kg), Cystone (750 mg/kg), or PHF (375 mg/kg). Urine, serum, and kidney analyses, including LDH, GSH, LPO, and histopathology, were performed ($p<0.001$). **Results:** Methanolic extracts significantly reduced elevated biochemical markers (creatinine, calcium, phosphorus, uric acid, and alkaline phosphatase), restored urinary pH, and increased urine volume. PHF exhibited the highest efficacy, outperforming individual extracts. Antioxidant activity was enhanced, reducing kidney stone formation. *N. nucifera* showed the most potent activity among single extracts. **Conclusion:** The phytoconstituents, particularly saponins, played a key role in reducing calcium oxalate crystal formation and recurrence. This study highlights polyherbal formulations as cost-effective and efficient alternatives for managing kidney stones.

Keywords: Calcium oxalate, *Nelumbo nucifera*, *Anogeissus latifolia*, *Leucas aspera*, Saponins.

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Received: 26-11-2024;
Revised: 06-01-2025;
Accepted: 15-04-2025.

INTRODUCTION

Kidney stones are one of the most common nephrological disorders (Rodriguez Cuellar *et al*., 2020). Kidney stones recur due to urinary tract formation, causing pain, hematuria, and hydronephrosis. Prevalence (16% globally) varies by diet, lifestyle, location, sex, and genetics, with India having the highest incidence (Madsen 2013; Guha *et al*., 2019). About 70-80% of kidney stones consist of calcium phosphates and oxalates, with 1-5% prevalence in Asia. They form via crystal nucleation and aggregation of calcium, magnesium, phosphates, cystine, and uric acid, affecting both genders equally (Öner *et al*., 2015; Teo *et al*., 2021; Edvardsson *et al*., 2022). In modern times, kidney stone treatment often involves complex, sophisticated, and expensive surgical procedures. However, herbal remedies provide a cost-effective and widely accepted alternative (Papatsoris *et al*., 2024). Traditional medicinal plants, particularly in Ayurveda, have long been used to prevent crystallization, nucleation, and aggregation of urinary calculi, offering therapeutic benefits in the management of urolithiasis along with other pharmacological advantages (Rukari *et al*., 2024). The lotus, native to Asia, is revered as a sacred plant in traditional cultures. In tropical regions, it features a whip-shaped rhizome that retains its green coloration and continues to flower throughout its lifecycle (Cao *et al*., 2021). The lotus contains proteins, carbohydrates, and sugars (arabinose, galactose, mannose, xylose, rhamnose, glucuronic acid). It is rich in magnesium, calcium salts of ghattic acid, and phytochemicals like alkaloids, flavonoids, phenols, terpenoids, sterols, saponins, tannins, coumarins, quinones, and ellagic acid (Khan *et al*., 2024). Extensive phytochemical screening has successfully identified and extracted key bioactive compounds, particularly saponins, in significant proportions (Dalmarco, *et al*., 2010). The lotus contains vitamins, lipids, glycosides, and essential oils. *Leucas aspera* (Lamiaceae) derives its name from the Greek "Leukos" (white) and is widespread in India, especially the Himalayas, and Sri Lanka. The lotus is among the largest flowering plants. (Abdel-Alim *et al*., 2023). Another notable

DOI: 10.5530/jyp.20251729

species, *Anogeissus latifolia*, commonly known as Gum Ghatti, Axle-wood, or the Indian Gum Tree, belongs to the Combretaceae family (Singh *et al.*, 2023). It is widely distributed across Asian countries such as India, Sri Lanka, Myanmar, and Nepal, thriving in tropical and subtropical regions (Mahawar *et al.*, 2024). This study aims to evaluate the *in vitro* and *in vivo* anti-urolithiatic activity of NNE, ALE, LAE, and PHF, focusing on the extraction and pharmacological significance of saponins.

MATERIALS AND METHODS

Collection of Plant Material and Preparation of Extract

N. nucifera, A. latifolia, and *L. aspera* were collected from Sri Venkateswara University, Tirupati, and authenticated by Dr. K. Madhava Chetty (IDs: 1078, 1080, 1088). Plants were air-dried for seven days in the shade.

Chemical Agents and Apparatus Required

Calcium Oxalate-AR, (Merck, India), and Cystone (Himalaya Company Limited, India) are selected for the practical studies. If needed selection of the required chemicals and reagents were included with analytical grades. Auto analyzer Semi followed by UV- spectrophotometer UV-1700 Pharma-spec, Shimadzu.

Animals

Male Wistar rats (150-200 g) were procured and acclimatized to laboratory conditions before the study. They were housed in a controlled environment with regulated temperature, relative humidity, and hygienic conditions. Standard feed and water were provided *ad libitum* in accordance with quarantine protocols. Animal experiments followed CPCSEA guidelines and were approved by IAEC, Jeeva Life Sciences (CPCSEA/IAEC/JLS/18/07/22/029), adhering to ethical regulations.

Acute-toxicity studies

Toxicity studies were conducted according to Organization for Economic Cooperation and Development rules and regulations (Sewell *et al.*, 2024).

Calcium oxalate-induced urolithiasis rat model

To induce urolithiasis in rats, all groups (except the control group) were fed a commercially available pelleted diet with drinking water containing calcium oxalate (70 mg/kg, intraperitoneally) for 28 days (Table 1). Renal and serum biochemical parameters, along with histopathological studies, were subsequently performed (Ćorić-Martinović *et al.*, 2024).

Kidney's Histological Studies

Histopathological evaluation was carried out using a microtome. The kidneys were isolated, fixed in formalin (10%), and embedded in paraffin for 5 mm thick sections. Sections measuring 5 mm thick were stained with hematoxylin and eosin. The specimen was examined under a microscope (Li *et al.*, 2023).

Table 1: Treatment schedule.

Group No.	Group Name	Treatment
Normal	Normal control	Vehicle for 28 days.
Control	Disease control	Water with Calcium oxalate 70 mg/kg/i.p was given to the individual rats for 28 days.
Standard	Standard group	Cystone (750 mg/kg, orally) is given from the 14th to the 28th day with Calcium oxalate 70 mg/kg in drinking water.
Group 1	Test group 1	*NNE* (250 mg/kg, orally) is given from the 14th to the 28th day with Calcium oxalate 70 mg/kg in drinking water.
Group 2	Test group 2	*NNE* (500 mg/kg, orally) is given from the 14th to the 28th day with Calcium oxalate 70 mg/kg in drinking water.
Group 3	Test group 3	*ALE* (250 mg/kg, orally) is given from the 14th to the 28th day with Calcium oxalate 70 mg/kg in drinking water.
Group 4	Test group 4	*ALE* (500 mg/kg, orally) is given from the 14th to the 28th day with Calcium oxalate 70 mg/kg in drinking water.
Group 5	Test group 5	*LAE* (250 mg/kg, orally) is given from the 14th to the 28th day with Calcium oxalate 70 mg/kg in drinking water.
Group 6	Test group 6	*LAE* (500 mg/kg, orally) is given from the 14th to the 28th day Calcium oxalate 70 mg/kg in drinking water.
Group 7	Test group 7	*PHF* (375 mg/kg, orally) is given from the 14th to the 28th day with Calcium oxalate 70 mg/kg in drinking water.

NNE, ALE, LAE, and PHF: *Nelumbo nucifera, Anogeissus latifolia, Leucas aspera*, and polyherbal formulation.

Statistical Analysis

All the results were scrutinized by analysis of variance (Dunnett's comparison test) using Prism (v8.2) and articulated as mean and SD. The significance ($p<0.05$) was considered.

RESULTS

Preliminary phytochemical screening Evaluation

Preliminary phytochemical evaluation revealed the presence of supportive active phyto-constituents such as alkaloids, flavonoids, tannins, phenols, saponins, phytosterol, terpenoids, and glycosides (Table 2).

Acute oral toxicity

In acute oral toxicity evaluated on selected rats (both sexes) for 14 days, no signs and symptoms of toxicity were noticed at the limit-test dose of 2000 mg/kg administered as per the OECD guidelines.

Anti-urolithiatic activity on Calcium oxalate induced rat model

Methanolic extracts of NNE, ALE, LAE, and PHF improved creatinine, calcium, phosphorus, uric acid, and ALP in CaOx-induced rats. Ethylene glycol raised calcium, magnesium,

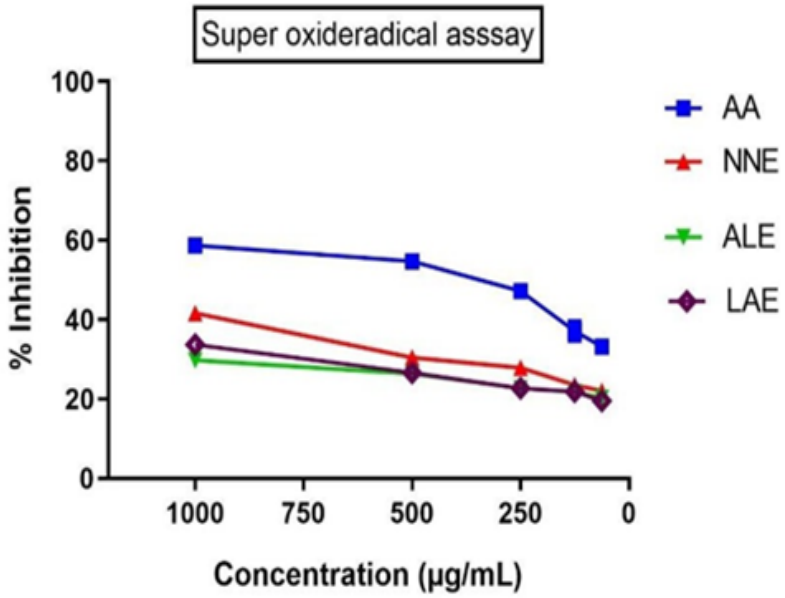

Figure 1: Effect of extracts on *in vitro* antioxidant activity. *$p<0.05$, **$p<0.01$, ***$p<0.01$. Values are mean±SEM, $n=6$, when compared with control by using one-way ANOVA followed by Tukey's multiple comparison test. ANOVA: Analysis of variance, SEM: Standard error of the mean, *NNE, ALE, LAE, and PHF*: *Nelumbo nucifera, Anogeissus latifolia, Leucas aspera,* and *PHF* extract.

Table 2: Preliminary phytochemical results for selected plant extracts.

		Results		
Sl. No.	Phytochemicals	NNE	ALE	LAE
1.	Alkaloids	-	+	+
2.	Flavonoids	+	+	+
3.	Tannins	+	+	+
4.	Phenols	+	+	+
5.	Carbohydrates	+	+	+
6.	Saponins	+	+	+
7.	Phytosterol	+	+	-
8.	Terpenoids	+	+	+
9.	Glycosides	+	+	+
10.	Anthraquinones	-	-	-

(+) Present; (-) Absent; NNE, ALE, LAE, and PHF: *Nelumbo nucifera, Anogeissus latifolia, Leucas aspera*, and polyherbal formulation.

and phosphate in the disease group, while extracts reduced these levels. PHF showed superior efficacy, also increasing oxalate levels more effectively than high-dose extracts (Tables 3, 4 and Figure 1).

In vitro anti-urolithic activity

Titrimetric analysis measured calcium oxalate stone dissolution. *Nelumbo nucifera* and *Leucas aspera* showed 80% dissolution, while *Anogeissus latifolia* achieved 74%, compared to the blank and positive control (Neeri) (Figure 2). This demonstrates that selected plants showed significant anti-urolithiasis activity *in vitro.* These plants can be used effectively to treat urolithiasis.

Calcium oxalate-induced renal stones caused crystal deposits, tubular atrophy, cystic changes, and inflammation. Cystone (70 mg/kg) and plant extracts (250-500 mg/kg, 28 days) significantly reduced these abnormalities (Figure 3). CaOx treatment significantly (p<0.01) reduced urine volume without altering pH. Cystone and plant extracts (250-500 mg/kg) increased urine volume (p<0.05-0.01) compared to controls, with no pH change. Similarly, rats treated with *N. nucifera* (NNE), *A. latifolia* (ALE), *L. aspera* (LAE), and PHF at doses of 250, 500, and 375 mg/kg body weight showed a significant (p<0.05 to 0.01) increase in urine volume, without affecting urine pH (Figure 4).

CaOx (70 mg/kg, i.p.) induced hyperoxaluria, altering urinary markers and causing tubular damage. Cystone (500-750 mg/kg) and plant extracts (250-500 mg/kg) restored levels, reduced oxidative stress, and prevented lithiasis. PHF was most effective. Histological analysis showed reduced tubular damage and crystal deposits with treatment.

DISCUSSION

In the current study, the calcium oxalate-induced model in Wistar rats was used as a representation of the human urinary system (Wang *et al.*, 2025). Previous research has shown that renal crystal deposition is more pronounced in male rats compared to females (Peeters *et al.*, 2022; Shi, *et al.*, 2025). Calcium Oxalate (CaOx) renal stone formation begins with the deposition of calcium phosphate plaques, also known as Randall's plaques. These plaques attract cations, which, in turn, form insoluble calcium oxalate salts that deposit on the renal interstitium. The oxalate crystals then attach to renal cells, damaging cell walls, promoting crystal aggregation, and facilitating further crystal growth, ultimately leading to retention in the renal tubules (Kaur *et al.*, 2025; Chung H.J. 2014; Kostov *et al.*, 2025). NNE, ALE, LAE, and PHF reduced urinary oxalate levels and LDH activity, lowering oxalate accumulation. CaOx crystals increased LPO and oxidative stress, leading to kidney damage. Treated groups showed reduced urine output and filtration rates, causing nitrogenous waste buildup (Ran *et al.*, 2025; Kanlaya *et al.*, 2024). Treatments with PHF reduced the deposition of calcium oxalate crystals and lowered the levels of creatinine, phosphorus, and uric acid. The extracts of NNE, ALE, and LAE also decreased calcium oxalate

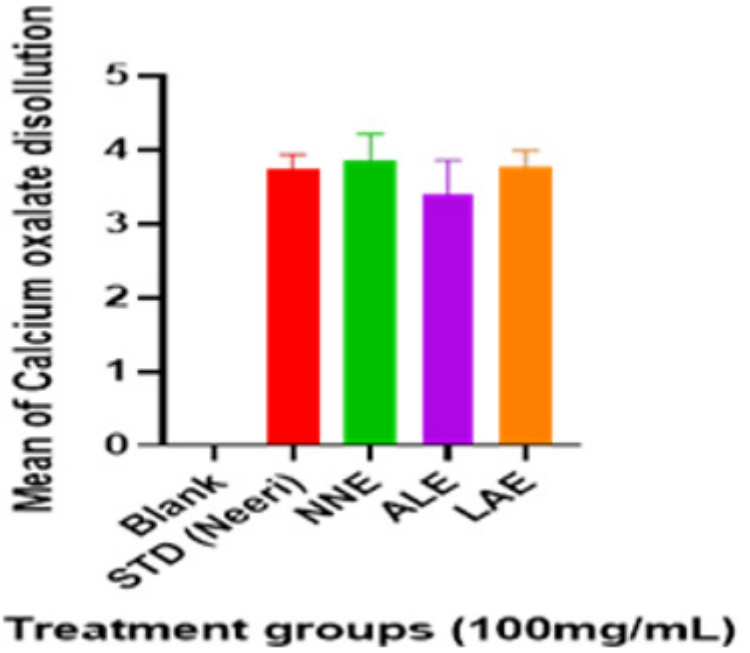

Figure 2: Effect of extracts on *in vitro* anti-urolithiasis activity. *p<0.05, **p<0.01, ***p<0.01. Values are mean±SEM, n=6, when compared with control by using one-way ANOVA followed by Tukey's multiple comparison test. ANOVA: Analysis of variance, SEM: Standard error of the mean, *NNE, ALE, LAE*, and *PHF*: *Nelumbo nucifera, Anogeissus latifolia, Leucas aspera,* and *PHF* extract.

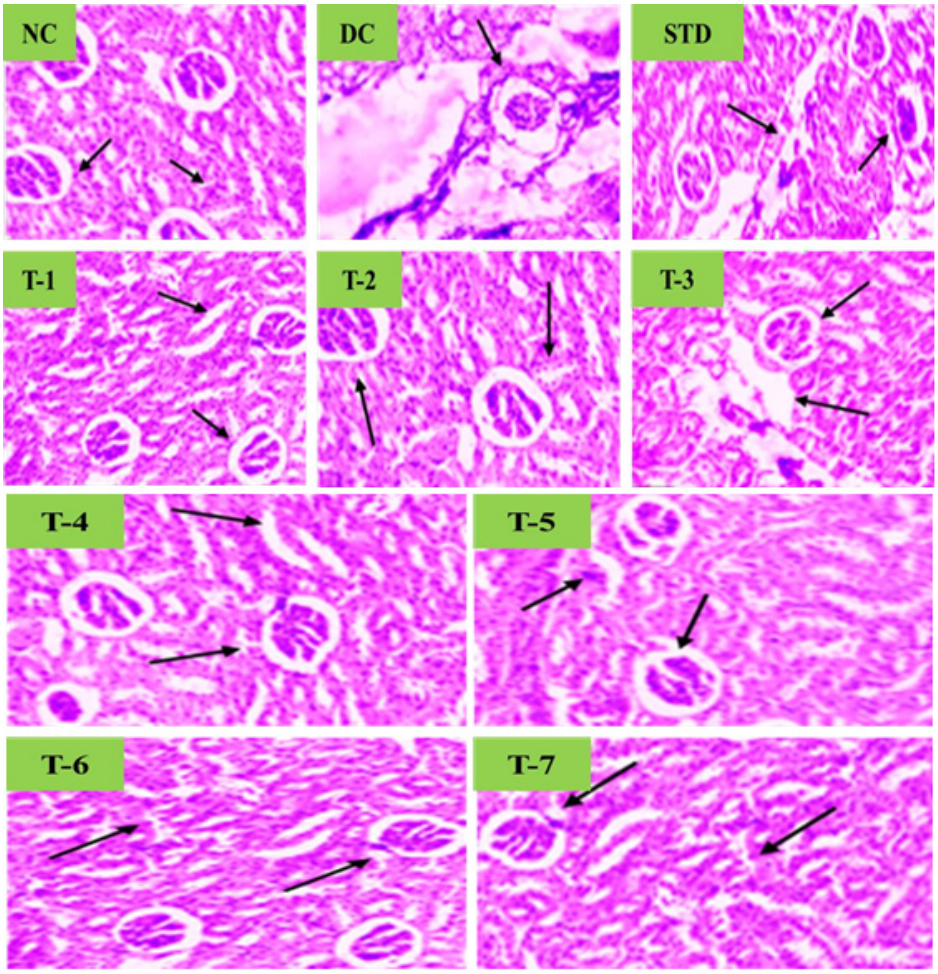

Figure 3: Effect of selected plant extracts on Calcium oxalate-induced Urolithiasis Rats on kidney tissue histology.

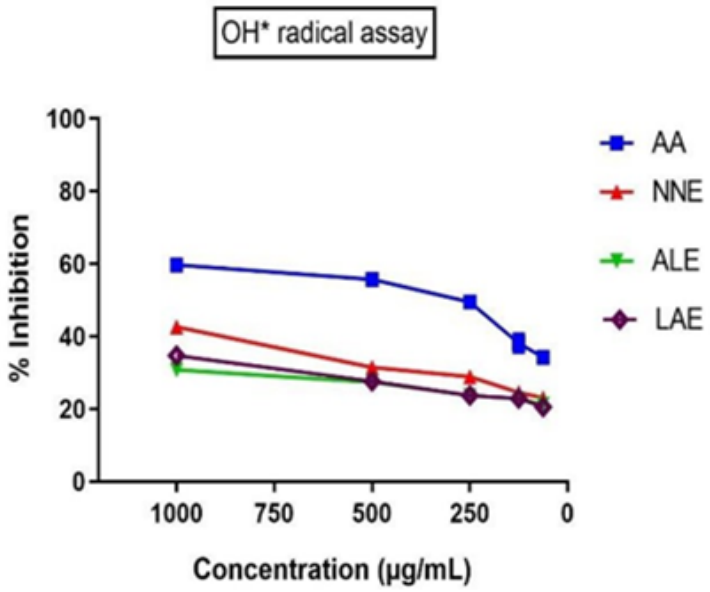

Figure 4: Hydroxyl (OH•) radical scavenging assay. The graph depicts the percentage inhibition of hydroxyl radicals at varying concentrations (1000-250 µg/mL) for AA (Ascorbic Acid), NNE (sample), ALE (sample), and LAE (sample). Data points represent mean values, showing a dose-dependent inhibition trend.

Table 3: Effect of extracts on *in vitro* antioxidant activity.

Concentration (Î¼g/mL)	DPPH Assay (AA)	DPPH Assay (NNE)	DPPH Assay (ALE)	DPPH Assay (LAE)	OH* Radical Assay (AA)	OH* Radical Assay (NNE)	OH* Radical Assay (ALE)	OH* Radical Assay (LAE)	Superoxide Radical Assay (AA)	Superoxide Radical Assay (NNE)	Superoxide Radical Assay (ALE)	Superoxide Radical Assay (LAE)
1000	61.81±0.24	44.7±0.26	36.84±0.53	32.91±0.76	59.72±0.21	42.6±0.23	30.81±0.23	34.74±0.53	58.72±0.45	37.49±0.52	29.81±0.53	33.74±0.63
500	57.78±1.02	33.52±0.55	29.74±0.70	29.48±0.75	55.69±0.23	31.42±0.52	27.38±0.56	27.64±0.48	54.69±0.56	29.12±0.45	26.38±0.35	26.64±0.25
250	51.25±0.096	30.92±0.77	25.77±0.46	25.77±0.46	49.48±0.53	28.82±0.25	23.67±0.53	23.67±0.56	47.19±0.42	26.16±0.41	22.67±0.12	22.67±0.53
125	40.21±2.31	26.64±0.42	24.96±0.71	24.86±0.40	38.11±0.25	24.54±0.23	22.76±0.58	22.86±0.86	37.11±0.53	22.94±0.12	21.76±0.13	21.86±0.36
62.5	36.31±0.36	25.18±0.29	22.6±0.86	23.42±0.40	34.21±0.15	23.07±0.56	21.32±0.42	20.5±0.12	33.21±0.52	21.67±0.23	20.32±0.63	19.5±0.31
IC50 (Î¼g/mL)	82.25±0.23	85.56±0.15	75.25±0.45	68.56±0.52	86.32±0.23	82.25±0.35	76.22±0.65	58.56±0.85	80.25±0.52	78.46±0.19	73.25±0.49	62.56±0.45

*p<0.05, **p<0.01, ***p<0.01. Values are mean±SEM, n=6 when compared with control using one-way ANOVA followed by Tukey's multiple comparison test. ANOVA: Analysis of variance, SEM: Standard error of the mean, NNE, ALE, LAE, and PHF: *Nelumbo nucifera, Anogeissus latifolia, Leucas aspera*, and PHF extract.

Table 4: Effect of extracts on *in vitro* anti-urolithiasis activity.

	Results				
Groups	Weight of Calcium Reduced (mg)				
	Test 1	Test 2	Test 3	MEAN	% Dissolution
Blank	5	5	5	5.00	0
STD (*Neeri*)	3.8	3.9	3.5	4.01***	80.26
NNE (100 mg/mL)	3.9	4.2	3.5	4.11***	82.29
ALE (100 mg/mL)	2.9	3.8	3.5	3.73***	74.53
LAE (100 mg/mL)	3.5	3.9	3.9	4.04***	8.78

deposition, minimizing damage to the urinary system (Simhadri and Leslie, 2024).

The deposition of CaOx crystals causes renal and histological damage in various parts of the renal system. A microscopic study of kidney sections from lithiatic control rats showed CaOx crystals in tubular and interstitial spaces, accompanied by glomerular congestion and tubular necrosis. However, treatment with NNE, ALE, LAE, and PHF extracts resulted in a reduction of CaOx crystal deposition. High concentrations of CaOx can lead to renal tubular obstruction, glomerular impairment, and tubular necrosis (Kale *et al.*, 2024; Hou *et al.*, 2025; Raj *et al.*, 2024). The plant extracts showed stone-dissolution properties, likely due to their saponin content. NNE, ALE, and LAE contained saponins, flavonoids, and phytosterols, contributing to anti-lithiatic and antioxidant effects. These phytochemicals may prevent CaOx supersaturation, reducing crystal deposition. The antioxidant activity improved renal markers by inhibiting oxidative stress. The study confirms their inhibitory and preventive effects on urolithiasis (Mohamed *et al.*, 2024).

CONCLUSION

These studies conclude that the administration of *NNE, ALE, LAE*, and particularly *PHF* decreases urinary stone formation. Therefore, in the early stages of stone formation, *Nelumbo nucifera, Anogeissus latifolia*, and *Leucas aspera* plant extracts are recommended in the recurrent pattern of renal stone disease. The mechanism behind this effect may be due to antioxidant nephroprotective properties as well as a reduction in urinary stone-forming constituent concentrations. However, further research is needed to determine the precise mechanism of this behavior.

ACKNOWLEDGEMENT

I sincerely thank Dr. Subhranshu Panda, Director, Jaipur National University, Jaipur, Dr. G. Tharun Kumar, Professor, and Dr. N. Sriram Nagarajan for their support. I also extend my gratitude to the Principal, Teegala Krishna Reddy College of Pharmacy, Hyderabad, for guidance and library access, and to Jeeva Life Sciences Labs, Telangana, for providing laboratory resources and a safe research environment.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ALE: *Anogeissus latifolia* extract; **ALP:** Alkaline phosphatase; **AAT:** Alanine amino transferase; BUN: Blood urea nitrogen; **CPCSEA:** Committee for the purpose of control and supervision on experiments in animals; **DPPH:** 2,2-diphenyl-1-picrylhydrazyl; **GSH:** Glutathione Stimulating hormone; **IAEC:** Institutional Animal Ethics Committee; **LAE:** *Leucas aspera* extract; **LDH:**

Lactate dehydrogenase; **LPO:** Lipid peroxidation; **NNE:** *Nelumbo nucifera* extract; **OECD:** Organization for economic cooperation and development; **PHF:** Polyherbal formulation; **SD:** Standard deviation.

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Cite this article: Panda S, Erigi NC, Gaddapuram TK. *In vitro* Anti-Urolithiatic Evaluation of a Polyherbal Formulation against Calcium Oxalate-Induced Urolithiasis in Rats. J Young Pharm. 2025;17(2):470-6.