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IN VITRO AND *IN VIVO* EVALUATION OF THROMBOLYTIC AND ANTI - UROLITHIATIC ACTIVITY OF *PIPER BETEL* LEAF EXTRACT WITH STANDARD DRUGS

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Abstract:

The present study was designed to evaluate the in vitro and in vivo anti-urolithiatic and thrombolytic activities of Piper betel leaf extract using standard experimental models. Urolithiasis was induced in rats using ethylene glycol (EG) administration, while thrombosis was induced using the ferric chloride ($FeCl_3$) model. In vitro anti-urolithiatic activity was assessed by calcium oxalate crystallization inhibition and struvite crystal growth models, whereas in vitro thrombolytic activity was evaluated using clot lysis assays. In the EG-induced urolithiasis model, Piper betel extract significantly reduced urinary calcium and oxalate levels, serum creatinine, and kidney weight in a dose-dependent manner. In vitro studies showed significant inhibition of calcium oxalate crystallization and crystal growth, indicating interference with crystal formation and aggregation processes. In the $FeCl_3$-induced thrombosis model, the extract significantly reduced thrombus weight and improved platelet count. In vitro clot lysis studies further demonstrated a marked increase in percentage clot lysis and reduction in clot dissolution time in a concentration-dependent manner.

The observed pharmacological activities may be attributed to the presence of bioactive phytoconstituents such as flavonoids, phenolic compounds, tannins, and alkaloids, which possess antioxidant, anti-inflammatory, and membrane-stabilizing properties. Overall, the findings suggest that Piper betel leaf extract exhibits significant anti-urolithiatic and thrombolytic activities, thereby supporting its traditional medicinal use and indicating its potential as a natural therapeutic agent. Further studies are recommended to isolate active compounds and elucidate the exact mechanism of action.

Keywords: *Piper betel, anti-urolithiatic activity, thrombolytic activity, calcium oxalate crystallization, struvite crystals.*

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INTRODUCTION:

Since humankind's earliest days, plants have been used as a source of medical compounds. In the past, all medications were derived from natural materials. People are returning to nature and medicinally valuable natural goods produced from herbs, shrubs, and trees at a time when science and technology have advanced significantly in every field and synthetic products are appearing at a rapid pace. The World Health Organization estimates that in developing countries, 80 percent of the population uses medications derived from plants. Unani, Ayurveda, Siddha, and homoeopathy are all examples of Indian medical systems that saw widespread use in the country. In addition to East Asian and African countries, they are also utilized in several other nations worldwide. The rich diversity and information of our country's traditional medical systems provide the pharmaceutical industry with the inspiration and competitive advantage to develop flavouring, mineral, and polyhedral formulations. Indian Systems of Medicine assert that they have treatments for resistant disorders such as inflammatory disease, epilepsy, respiratory problem, liver condition, etc. with no or minimal side effects. Synthetic products briefly overtook them in the modern age. Others, in search of safety and security, are returning to natural substances since the mindless dependence on synthetics has ended. More than seventy-five percent of the global population uses plants or plant extracts as their primary source of medical care. There are still many people who use conventional methods of healthcare. Plant materials are under increasing pressure to be used as a source of medicines for a wide range of human diseases due to factors such as population growth, inadequate medicine supply, the preventive value of treatments, the adverse effects of many allopathic medications, and the emergence of resistance to currently available antibiotics for infectious diseases. Global estimates suggest that 80% of the world's four billion people rely on traditional remedies made primarily from plants since they cannot afford Western pharmaceutical items [1-2].

The almost 20,000 plant species in the medicinal plant list witness to this. Despite the dominating influences of modern drugs and our dependence on them, as well as the enormous developments in synthetic medicine, a significant portion of the world's population continues to flavour plant-based medicines. Despite the fact that many developing countries devote between 40 and 50% of their GDP to pharmaceuticals and health care, modern life-saving treatments are out of reach for three-quarters of the people in the third world. To help lessen the economic load on poor countries, there will soon be a greater emphasis on the use of plant medicine.

Plants, especially those used in Ayurvedic medicine, will yield physiologically active chemicals and lead structures for the creation of derivatives with improved activity and/or reduced toxicity. Secondary product examples include pigments, alkaloids, isoprenoids, terpenes, and waxes. For thousands of years, secondary plant materials have played a critical role in medicine. Historically, they must have been consumed and utilized as herbs. They are utilized either directly or after undergoing a chemical transformation. Secondary metabolites of plants are an enormous resource for scientific and clinical study, as well as the development of new medications. Overall, their pharmacological usefulness has not only persisted to the present day but is increasing as their prospective applications in healthcare and as a lead chemical for the development of novel medications are more recognized. Products of secondary metabolism in plants are extremely diverse, but can be broken down into several groups. These include alkaloids, glycosides, isoprenoids, terpenes, plant amines, phenolic compounds, polyisoprene-like chemicals, and uncommon amino acids. The majority of natural plants are utilized for dye, tannin, gum, resins, agricultural and seeking equipment, weaponry, and witchcraft, in addition to their traditional medicinal applications. Similarly, it has come to light that a number of the current disease-curing instruments are blunt, unwieldy, and expensive. By adopting costly modern medicine and technology, we have made significant improvements in health care. A generally acceptable alternative would be to bring health services and facilities to the agricultural population by encouraging them to utilize what is currently available. In their environment and what they are acquainted with. In ancient times, crude extracts or juices derived from India's medicinal plants were utilized. Ayurveda and Siddha formulations replaced the use of crude extracts as civilization and the field of medicine advanced. Plants utilized in Ayurveda and Siddha remedies have a low lethal dose [3].

Consequently, these formulations can be employed alone or in conjunction with other drugs to achieve the desired therapeutic impact with minimal lethal side effects. In addition, these formulations are taken orally. Consequently, the systemic toxicity of the substance is reduced. In addition, these formulations keep the advantages of the oral mode of administration. In the nineteenth century, advancements were made at a faster clip. The first major alkaloid, narcotine, was discovered in 1803. Many more, including morphine, strychnine, emetine, and many others, were discovered between 1813 and 1823. Chevreul demystified the chemistry behind oils and fats. Before the middle of the twentieth century, natural product chemistry mostly focused on isolating and determining the structures of various chemicals. Even now, when the vast

majority of the most crucial pathways and stereo chemical characteristics are investigated at length, this emphasis remains constant. The field of comparative photochemistry has also recently seen a rise in popularity. Herbal treatments have been utilized for hundreds of years, but synthetic organic chemists may now produce the molecules in-vitro and hence on a bigger scale. An estimated one-fourth of prescribed medications contain at least one constituent originally identified and derived from a plant. Currently, it is understood that numerous plant elements, particularly those associated with herbal remedies, have medicinal characteristics that appear more subtly and less spectacularly than overtly hazardous species. As a result, a much wider variety of plant metabolites are now being considered for in-depth study. Other nutrient groups, such as carbohydrates, lipids, and proteins, are of nutritional relevance in addition to components like calcium oxalate, silica, lignin, and colouring pigments, which help in the identification of medications and the detection of adulteration. Therefore, it is crucial to conduct scientific studies of traditional medicines, derive medications through bioprospecting, and systematically conserve the involved medicinal plants. [4-5]

Methodology

Plant collection and authentication

Betel leafs Used for This Study Were Collected from local market Telangana and authenticated.

Preparation of Extract

Fresh leaves of *Piper betel* were collected, washed thoroughly with distilled water, and fresh leaves were powdered using a mechanical grinder and obtain coarse powder. About 50 g of the powdered material was packed in a thimble and extracted with 250 mL of ethanol using a Soxhlet apparatus. The extraction process was continued for 6–8 hours until the solvent in the siphon tube became colourless. The obtained extract was filtered and concentrated using a rotary evaporator or water bath at controlled temperature. The concentrated extract was then dried and stored in an airtight container for further pharmacological evaluation.

Experimental Animals

Healthy adult Wistar albino rats of either sex weighing about 150–250 g are commonly used for *in vivo* anti-urolithiatic studies. The animals are maintained under standard laboratory conditions at room temperature (25 ± 2°C) with relative humidity of 45–55% and a 12-hour light/dark cycle. The rats are provided with standard pellet diet and water ad libitum. Before the experiment, animals are acclimatized to laboratory conditions for at least one week. Experimental protocols are carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA

In Vivo Anti-Urolithiatic Activity:

Ethylene Glycol Model in Rats: The in vivo anti-urolithiatic activity of *Piper betel* leaf extract is evaluated using the ethylene glycol (EG)-induced urolithiasis model in Wistar rats. Healthy adult Wistar rats (180–250 g) are randomly divided into different groups such as normal control, urolithiatic control, standard drug, and test extract-treated groups. Urolithiasis is induced by administering 0.75% ethylene glycol (v/v) in drinking water for 21 days to all groups except the normal control. In addition, 1% ammonium chloride (w/v) is administered orally for the initial 3–5 days to accelerate stone formation by inducing metabolic acidosis and increasing urinary oxalate excretion. From the 7th day onwards, the standard drug such as Cystone and *Piper betel* leaf extract at selected doses are administered orally once daily and continued up to the 21st day along with ethylene glycol treatment.

During the experimental period, animals are maintained under standard laboratory conditions with free access to food and water. On the 21st day, rats are placed in metabolic cages for 24-hour urine collection for analysis of urine volume, calcium, oxalate, phosphate, and uric acid levels. After urine collection, blood samples are collected for estimation of serum creatinine, urea, and blood urea nitrogen (BUN). The animals are then sacrificed humanely, and both kidneys are carefully removed, washed with normal saline, blotted dry, and weighed to determine the kidney weight index. One kidney is used for biochemical estimation of renal calcium and oxalate content, while the other is preserved in 10% formalin for histopathological examination to observe calcium oxalate crystal deposition, tubular damage, and restoration of normal renal architecture. A significant improvement in urinary, serum, and renal parameters in treated groups compared to the urolithiatic control group indicates the anti-urolithiatic potential of the test extract.

Group Treatment

Group I Normal control (normal feed and water)
Group II Disease control (0.75% ethylene glycol)
Group III Standard treatment + ethylene glycol + Cystone (750 mg/kg, p.o.)
Group IV Ethylene glycol + Piper betel leaf extract (200 mg/kg, p.o.)
Group V Ethylene glycol + Piper betel leaf extract (400 mg/kg, p.o.)

Invivo thrombolytic activity

$FeCl_3$-induced thrombosis model: The Ferric Chloride ($FeCl_3$)-induced thrombosis model is a widely accepted in vivo method for evaluating the thrombolytic activity of *Piper betel* leaf extract. Healthy adult Wistar rats (180–250 g) are divided into experimental groups, including normal control, thrombosis control, standard drug-treated, and

extract-treated groups. The animals are anesthetized using ketamine and xylazine, and the common carotid artery is surgically exposed through a midline neck incision. A small filter paper soaked in 10% $FeCl_3$ solution is applied directly to the artery for 5 minutes to induce endothelial damage and thrombus formation. After removing the filter paper, the area is washed with normal saline. The standard thrombolytic drug, such as Streptokinase (30,000 IU/kg), and the *Piper betel* leaf extract at selected doses are administered according to the study design. Following a treatment period of 24 hours, the animals are sacrificed, and the thrombosed arterial segment is excised. The thrombus is carefully isolated and weighed. Blood samples are collected for coagulation studies, including Prothrombin Time (PT), Activated Partial Thromboplastin Time (aPTT), and Thrombin Time (TT). Histopathological examination of the arterial tissue may also be performed to assess thrombus dissolution and vascular integrity. A significant reduction in thrombus weight and improvement in coagulation parameters compared with the thrombosis control group indicate the thrombolytic potential of the *Piper betel* leaf extract.

Experimental animal grouping:

Group I Normal Control (Vehicle)
Group II Thrombosis Control ($FeCl_3$-induced)
Group III Standard Drug + $FeCl_3$ (Streptokinase 30,000 IU/kg)
Group IV Piper betel Extract Low Dose (200 mg/kg) + $FeCl_3$
Group V Piper betel Extract High Dose (400 mg/kg) + $FeCl_3$

Determination of Thrombus Weight (mg) [6]

After completion of the treatment period in the $FeCl_3$-induced thrombosis model, the rats are anesthetized and sacrificed humanely. The carotid artery containing the thrombus is carefully exposed and excised. The arterial segment is opened longitudinally using fine surgical scissors, and the thrombus is gently removed with forceps without damaging the clot structure. The isolated thrombus is washed with normal saline to remove adhering blood and tissue debris, then blotted gently on filter paper to remove excess moisture. The thrombus is immediately weighed using a calibrated analytical balance, and the weight is recorded in milligrams (mg). The mean thrombus weight for each experimental group is calculated and expressed as Mean ± SEM. A significant reduction in thrombus weight in the extract-treated groups compared with the thrombosis control group indicates thrombolytic activity.

Calculation of Percentage Thrombus Reduction

$$\text{Percentage Thrombus Reduction} = \frac{\text{Mean Thrombus Weight of Control Group} - \text{Mean Thrombus Weight of Treated Group}}{\text{Mean Thrombus Weight of Control Group}} \times 100$$

Platelet Count:

Blood samples are collected from experimental rats at the end of the treatment period through retro-orbital puncture or cardiac puncture into EDTA-coated tubes to prevent clotting. The collected blood is mixed gently and analysed using an automated hematology analyzer for platelet estimation. For manual counting, 20 µL of whole blood is diluted with platelet diluting fluid (1% ammonium oxalate solution) and allowed to stand for 10–15 minutes to lyse red blood cells. The diluted sample is then loaded into a Neubauer hemocytometer, and the platelets are counted under a light microscope at 400× magnification. The platelet count is expressed as the number of platelets per microliter ($\times 10^3/\mu L$) of blood. The mean platelet count for each experimental group is calculated and expressed as Mean ± SEM (n = 6). An increase or normalization of platelet count in treated groups compared to the thrombosis control group indicates a protective effect against thrombus formation and platelet consumption.

Invitro Anti-Urolithiatic activity: [7]

Inhibition of Crystallization:

The percentage inhibition of calcium oxalate crystallization is determined by a turbidimetric method using spectrophotometric analysis. In this method, calcium oxalate crystals are prepared by mixing equimolar solutions of calcium chloride and sodium oxalate under controlled laboratory conditions. The resulting suspension is used as the standard crystal system. The test sample (Piper betel extract at different concentrations) and standard drug (such as Cystone) are added separately to the reaction mixture containing calcium chloride and sodium oxalate solutions in a suitable buffer system (usually Tris buffer, pH 7.4). The mixtures are incubated at 37°C for a fixed period to allow crystal formation. After incubation, the turbidity of each sample is measured at 620 nm using a UV–Visible spectrophotometer. The optical density (OD) reflects the extent of crystal formation, where higher OD indicates greater crystallization and lower OD indicates inhibition. The percentage inhibition of crystallization is calculated by comparing the OD of treated samples with that of the control using the standard formula. All readings are taken in triplicate and expressed as Mean ± SEM. A higher percentage inhibition indicates stronger anti-urolithiatic activity of the test extract.

Calculation

$$\text{Percentage Inhibition of Crystallization} = \frac{\text{OD of Control} - \text{OD of Test}}{\text{OD of Control}} \times 100$$

Struvite Crystal Growth Model [8]

The struvite crystal growth model is an *in vitro* method used to evaluate the anti-urolithiatic activity of drugs and plant extracts against magnesium ammonium phosphate stones. In this method, struvite crystals are formed by mixing solutions containing magnesium, ammonium, and phosphate ions under controlled laboratory conditions. Different concentrations of the extract of Piper betel are added to the reaction mixture to study their inhibitory effect on crystal growth and aggregation. The mixture is incubated at room temperature or 37°C for a specified period to allow crystal formation. The formed crystals are then observed microscopically for changes in crystal size, morphology, and number. The anti-urolithiatic activity is determined by comparing the reduction in struvite crystal growth with the control and standard drug Cystone. This model is useful for studying the prevention of infection-induced urinary stones.

In Vitro Thrombolytic Activity [8]

In vitro thrombolytic activity is performed to evaluate the clot dissolving potential of drugs and plant extracts under laboratory conditions. In this method, blood collected from healthy volunteers is allowed to clot in pre-weighed microcentrifuge tubes. After clot formation, the serum is carefully removed and different concentrations of the test extract are added to the clot. The tubes are then incubated at 37°C for a specific period, usually 60–90 minutes. Following incubation, the released fluid is removed and the tubes are weighed again to determine the extent of clot dissolution. The thrombolytic activity of the test sample is compared with the standard drug Streptokinase and a negative control. The percentage of clot lysis is calculated to evaluate fibrinolytic activity.

Procedure for Percentage Clot Lysis Assay [9]

Fresh venous blood was collected from healthy human volunteers and transferred into pre-weighed sterile microcentrifuge tubes (0.5 mL/tube). The tubes were incubated at 37°C for 45 minutes to allow clot formation. After clot formation, the serum was carefully removed without disturbing the clot, and each tube was weighed again to determine the clot weight. Different concentrations of the test extract of Piper betel were added separately to the tubes containing the clot. The standard group received Streptokinase, while distilled water was used as negative control. All tubes were incubated again at 37°C for 90 minutes. After incubation, the released fluid was carefully removed and the tubes were reweighed. The difference in clot weight before and after treatment was used to determine clot lysis. The percentage of clot lysis was calculated using the following formula:

$$\% \, Clot \, Lysis = \frac{Weight \, of \, released \, clot}{Initial \, clot \, weight} \times 100$$

Time for Clot Lysis

The time for clot lysis is an important parameter used in *in vitro* thrombolytic studies to determine the duration required for complete or partial dissolution of a blood clot by a test sample. In this method, preformed blood clots are treated with different concentrations of the extract of Piper betel and incubated at 37°C. The clot is periodically observed for dissolution, and the time taken for visible clot breakdown or maximum clot lysis is recorded. A shorter clot lysis time indicates stronger thrombolytic activity. The activity of the extract is compared with the standard drug Streptokinase and negative control. This parameter helps evaluate the efficiency and speed of fibrinolytic action of the test sample.

Clot Weight Reduction [10-15]

Clot weight reduction is an important parameter used in *in vitro* thrombolytic studies to evaluate the clot dissolving ability of drugs and plant extracts. In this method, blood collected from healthy volunteers is allowed to clot in pre-weighed micro centrifuge tubes. After clot formation, the serum is carefully removed and the tubes are weighed again to determine the initial clot weight. Different concentrations of the extract of Piper betel are then added to the clot and incubated at 37°C for a specified period. After incubation, the released fluid is removed and the tubes are reweighed. The reduction in clot weight before and after treatment indicates the thrombolytic activity of the extract. Greater reduction in clot weight reflects higher fibrinolytic potential. The activity is compared with the standard drug Streptokinase and negative control.

RESULTS:

Percentage yield:

$$\text{Percentage of yield} = \frac{\text{Weight of crude extract}}{\text{Weight of dried material}} \times 100$$

$$\text{Percentage of yield} = \frac{26g}{200g} \times 100 = 13\%$$

50gms of drug dissolved in 250ml of solvent in 1:4 Ratio

Phytoconstituent results

S. No	Phytoconstituent	Result
1	Alkaloids	+
2	Flavonoids	+
3	Phenolic compounds	+
4	Tannins	+
5	Saponins	+
6	Glycosides	+
7	Terpenoids	+
8	Steroids	± (present in trace)
9	Carbohydrates	+
10	Proteins & amino acids	+

Table -1 phytochemical test

Invivo Anti -urolithiatic activity:

Ethylene Glycol (EG)-Induced Urolithiasis Model in Rats:

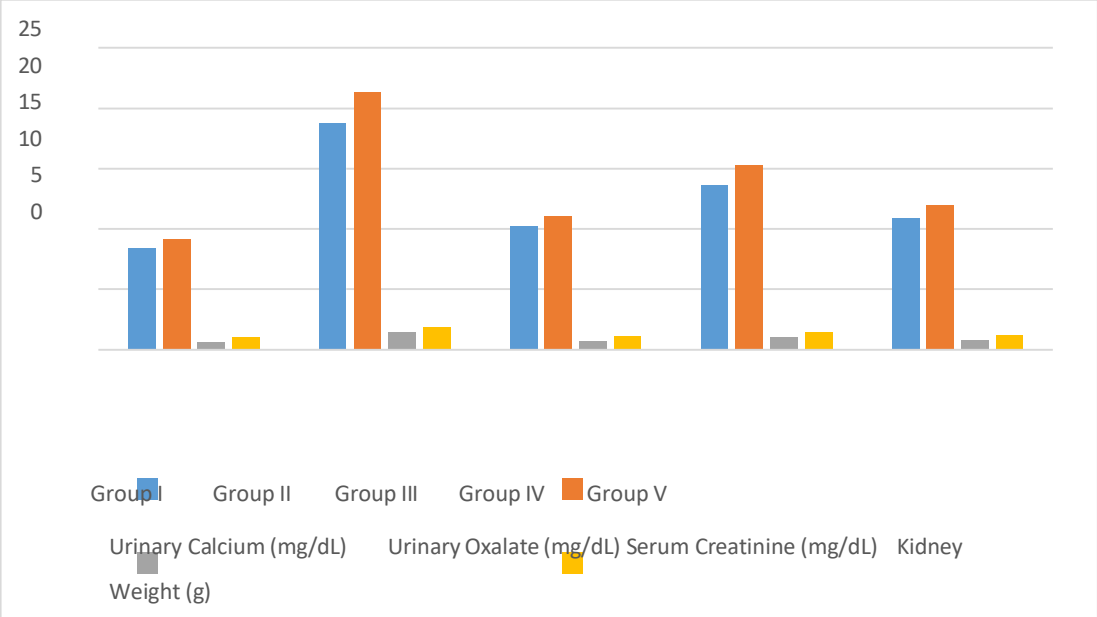
Group	Treatment	Urinary Calcium (mg/dL)	Urinary Oxalate (mg/dL)	Serum Creatinine (mg/dL)	Kidney Weight (g)
Group I	Normal Control	8.42 ± 0.31	9.15 ± 0.28	0.62 ± 0.03	1.02 ± 0.04
Group II	Urolithiatic Control (EG)	18.76 ± 0.52	21.34 ± 0.61	1.48 ± 0.06	1.85 ± 0.05
Group III	Cystone	10.21 ± 0.38***	11.03 ± 0.40***	0.71 ± 0.02***	1.11 ± 0.03***
Group IV	Piper betel Extract (200 mg/kg)	13.64 ± 0.44**	15.28 ± 0.47**	1.05 ± 0.04**	1.42 ± 0.04**
Group V	Piper betel Extract (400 mg/kg)	10.88 ± 0.36***	11.92 ± 0.39***	0.78 ± 0.03***	1.15 ± 0.03***

Table 2: Results for Ethylene glycol induced urolithiasis in rats

The ethylene glycol (EG)-treated urolithiatic control group showed a significant increase in urinary calcium and oxalate levels, along with elevated serum creatinine and increased kidney weight, indicating successful induction of renal stone formation and impaired renal function. Treatment with the standard drug Cystone significantly restored biochemical parameters toward normal levels. Piper betel leaf extract showed a dose-dependent protective effect. The low dose (200 mg/kg) moderately reduced urinary and serum markers, while the high dose (400 mg/kg) produced a significant reduction comparable to the standard drug. These findings indicate that Piper betel leaf extract possesses strong in vivo anti-urolithiatic activity by reducing crystal-forming constituents and improving renal function in ethylene glycol-induced urolithiatic rats.

Figure 1 : % Reduction of urolithiasis in different animal treated groups

Invivo thrombolytic activity:



Determination of Thrombus Weight (mg):

Group	Treatment	Thrombus Weight (mg)
Group I	Normal Control	0.00 ± 0.00
Group II	Thrombosis Control ($FeCl_3$)	21.84 ± 1.25
Group III	Streptokinase (30,000 IU/kg)	7.12 ± 0.68***
Group IV	Piper betel Extract (200 mg/kg)	13.96 ± 0.92**
Group V	Piper betel Extract (400 mg/kg)	9.34 ± 0.74***

Table 3: Thrombus weight reduction in different animal treated groups

The thrombosis control group showed a significant increase in thrombus weight (21.84± 1.25 mg) compared to the normal control group, confirming successful thrombus formation following $FeCl_3$ administration. Treatment with the standard drug, Streptokinase (30,000 IU/kg), markedly reduced thrombus weight to 7.12 ± 0.68 mg, indicating potent thrombolytic activity. Administration of *Piper betel* leaf extract produced a dose-dependent reduction in thrombus weight. The low dose (200 mg/kg) reduced thrombus weight to 13.96 ± 0.92 mg, while the high dose (400 mg/kg) further reduced thrombus weight to 9.34 ± 0.74 mg. These findings suggest significant thrombolytic activity of

Piper betel leaf extract, with the higher dose exhibiting effects comparable to the standard drug.

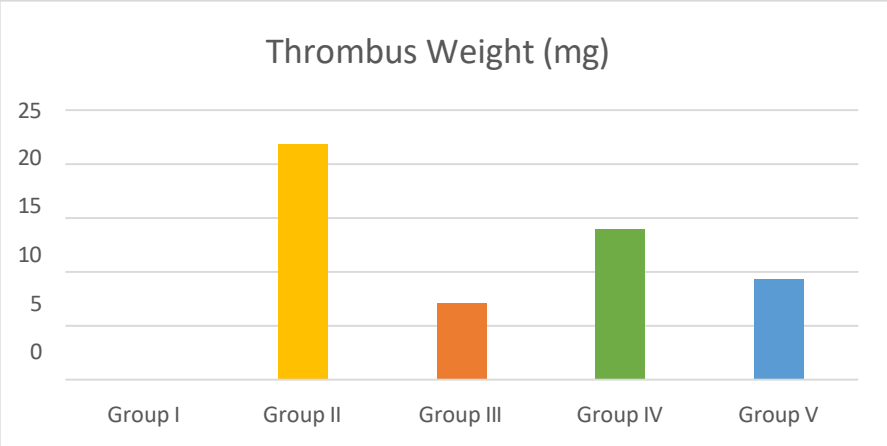


Figure 2: Thrombus weight reduction in different animal treated groups

Platelet Count:

Group	**Treatment**	**Platelet Count (×10³/µL)**
Group I	Normal Control	782.4 ± 24.6
Group II	Thrombosis Control ($FeCl_3$)	518.7 ± 18.3
Group III	Streptokinase (30,000 IU/kg)	741.6 ± 22.5***
Group IV	Piper betel Extract (200 mg/kg)	642.8 ± 20.7**
Group V	Piper betel Extract (400 mg/kg)	716.3 ± 21.4***

Table 4: Platelet count in different animal treated groups

The thrombosis control group exhibited a significant reduction in platelet count (518.7± 18.3 ×10³/µL) compared with the normal control group (782.4 ± 24.6 ×10³/µL), indicating increased platelet consumption during thrombus formation. Treatment with the standard drug, Streptokinase (30,000 IU/kg), significantly restored platelet levels to 741.6 ± 22.5 ×10³/µL. Administration of *Piper betel* leaf extract also produced a dose-dependent increase in platelet count. The low-dose group (200 mg/kg) showed a platelet count of 642.8 ± 20.7 ×10³/µL, while the high-dose group (400 mg/kg) exhibited a further increase to 716.3 ± 21.4 ×10³/µL. These findings indicate that *Piper betel* leaf extract effectively reduced platelet consumption associated with thrombosis and demonstrated significant thrombolytic activity, particularly at the higher dose.

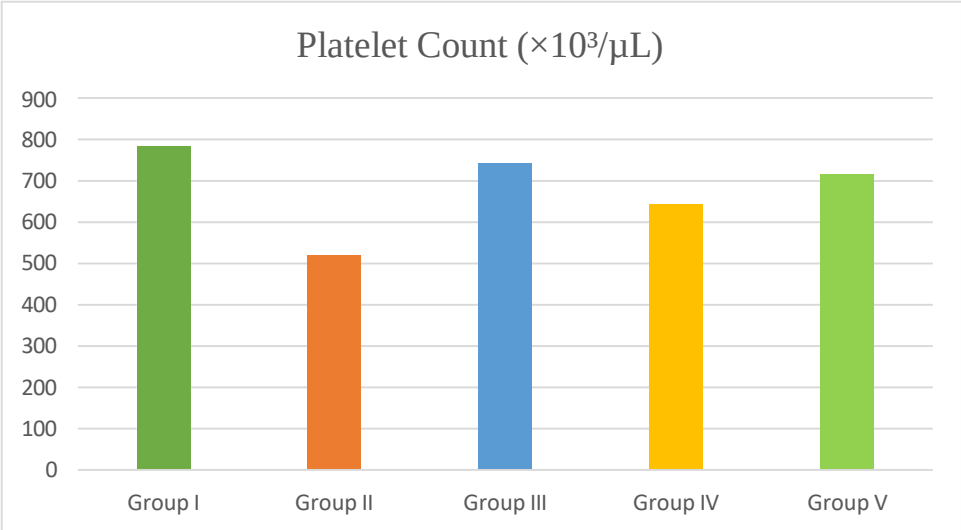


Figure 3: Platelet count in different animal treated groups

Invitro Anti- urolithiatic activity:

Inhibition of Crystallization (%):

Treatment	Concentration (µg/mL)	Absorbance (Mean ± SEM)	Inhibition of Crystallization (%) (Mean ± SEM)
Control	—	0.915 ± 0.014	0.00 ± 0.00
Standard (Cystone)	100	0.228 ± 0.008	75.08 ± 1.12
Piper betel Leaf Extract	25	0.764 ± 0.013	16.50 ± 0.92
Piper betel Leaf Extract	50	0.658 ± 0.011	28.09 ± 1.03
Piper betel Leaf Extract	100	0.548 ± 0.010	40.11 ± 1.07
Piper betel Leaf Extract	200	0.412 ± 0.009	54.97 ± 0.96
Piper betel Leaf Extract	400	0.296 ± 0.007	67.65 ± 0.89

Table 5: % Inhibition of Crystallization in Invitro Anti- Urolithiatic activity

The in vitro anti-urolithiatic activity of Piper betel leaf extract was evaluated by measuring its ability to inhibit calcium oxalate crystal formation. The untreated control exhibited the highest absorbance (0.915 ± 0.014) with 0% inhibition of crystallization, indicating maximum crystal formation. The standard drug, Cystone, showed the lowest absorbance (0.228 ± 0.008) and the highest inhibition of crystallization (75.08 ± 1.12%), demonstrating its potent anti-urolithiatic activity. The Piper betel leaf extract produced a concentration-dependent inhibition of calcium oxalate crystallization. As the concentration increased from 25 to 400 µg/mL, the absorbance progressively decreased from 0.764 ± 0.013 to 0.296 ± 0.007, while the percentage inhibition increased from 16.50 ± 0.92% to 67.65 ± 0.89%. The highest concentration (400 µg/mL) exhibited the maximum inhibition of crystallization, which was comparable to the standard drug, although slightly lower. These results indicate that Piper betel leaf extract effectively inhibits calcium oxalate crystal formation in vitro in a dose-dependent manner. The observed activity may be attributed to the presence of flavonoids, phenolic compounds, tannins, and other phytoconstituents that interfere with crystal nucleation, growth, and aggregation, thereby demonstrating promising anti-urolithiatic potential.

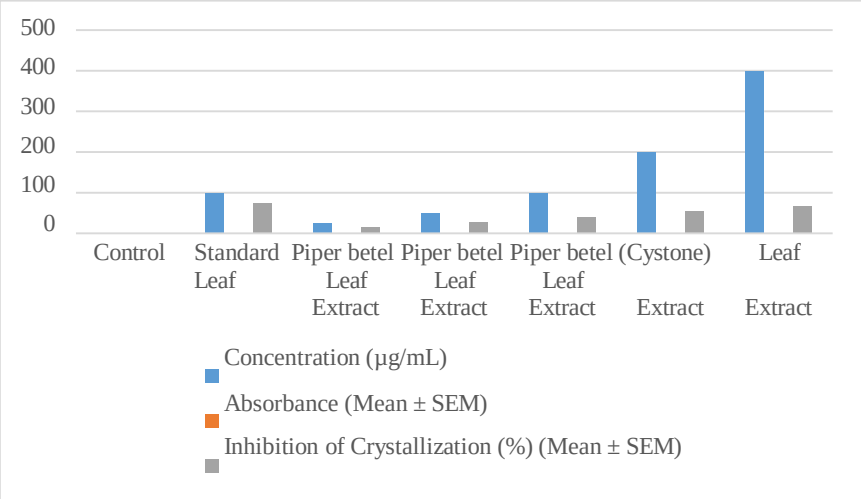


Figure 4: % Inhibition of Crystallization in Invitro Anti- Urolithiatic activity

Struvite crystal growth model

Treatment	Concentration (µg/mL)	Absorbance (Mean ± SEM)	Inhibition of Struvite Crystal Growth (%) (Mean ± SEM)
Control	—	0.928 ± 0.013	0.00 ± 0.00
Standard (Cystone)	100	0.214 ± 0.007	76.94 ± 1.05
Piper betel Leaf Extract	25	0.781 ± 0.012	15.84 ± 0.89
Piper betel Leaf Extract	50	0.664 ± 0.011	28.45 ± 0.95
Piper betel Leaf Extract	100	0.541 ± 0.010	41.70 ± 1.01
Piper betel Leaf Extract	200	0.398 ± 0.008	57.11 ± 0.92
Piper betel Leaf Extract	400	0.281 ± 0.007	69.72 ± 0.86

Table 6: % Reduction of Crystal growth in Struvite crystal growth model

The in vitro struvite crystal growth inhibition assay demonstrated that Piper betel leaf extract significantly inhibited the growth of struvite crystals in a concentration-dependent manner. The untreated control exhibited the highest absorbance (0.928 ± 0.013) with 0% inhibition, indicating maximum crystal growth. The standard drug, Cystone, showed the lowest absorbance (0.214 ± 0.007) and the highest inhibition of struvite crystal growth (76.94 ± 1.05%), confirming its potent anti-urolithiatic activity. Treatment with Piper betel leaf extract resulted in a gradual reduction in absorbance from 0.781 ± 0.012 at 25 µg/mL to 0.281 ± 0.007 at 400 µg/mL, accompanied by a corresponding increase in the percentage inhibition of crystal growth from 15.84 ± 0.89% to 69.72 ± 0.86%. The highest concentration (400 µg/mL) exhibited marked inhibition of struvite crystal growth, approaching the activity of the standard drug. Piper betel leaf extract possesses significant in vitro anti-urolithiaticactivity by effectively suppressing struvite crystal growth. The activity is likely attributable to its phytoconstituents, including flavonoids, phenolic compounds, tannins, and other antioxidants, which may interfere with crystal nucleation, growth, and aggregation, thereby reducing the formation of urinary stones.

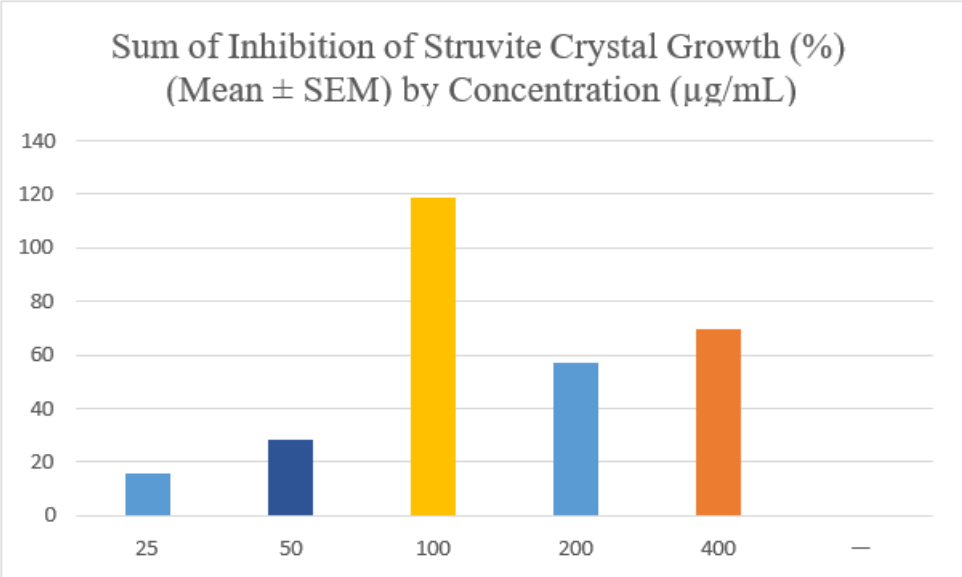


Figure 5: % Reduction of Crystal growth in Struvite crystal growth model

Invitro *thrombolytic activity:*

Clot Lysis Assay:

Treatment	**Concentration (µg/mL)**	**Absorbance (Mean ± SEM)**	**Clot Lysis (%) (Mean ± SEM)**
Control	—	0.846 ± 0.013	0.00 ± 0.00
Standard (Streptokinase)	100	0.224 ± 0.008*	73.52 ± 1.16*
Piper betel Leaf Extract	25	0.706 ± 0.012	16.55 ± 0.94
Piper betel Leaf Extract	50	*0.601 ± 0.011 **	*28.96 ± 1.02 **
Piper betel Leaf Extract	100	0.489 ± 0.009 **	42.20 ± 1.08 **
Piper betel Leaf Extract	200	0.371 ± 0.008*	56.15 ± 0.97*
Piper betel Leaf Extract	400	0.276 ± 0.007*	67.38 ± 0.89*

Table 7: Results for Invitro Thrombolytic activity by Clot lysis Assay

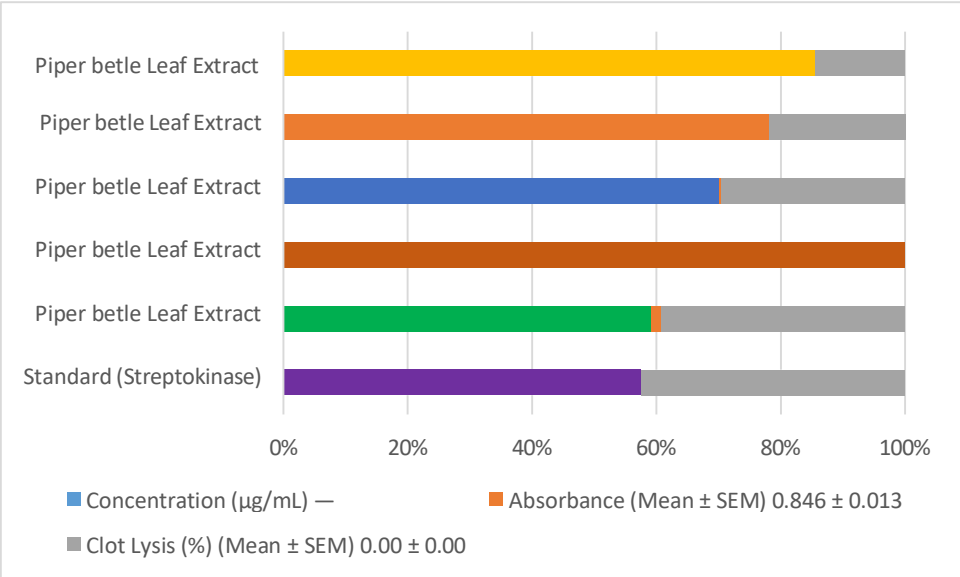


Figure 6: % Clot lysis in In vitro Thrombolytic Activity

Time taken for clot lysis:

Treatment	Concentration (µg/mL)	Time for Clot Lysis (min) (Mean ± SEM)
Control	—	120.00 ± 0.00
Standard (Streptokinase)	100	18.42 ± 0.76*
Piper betel Leaf Extract	25	96.84 ± 2.11
Piper betel Leaf Extract	50	*79.26 ± 1.84 **
Piper betel Leaf Extract	100	61.48 ± 1.56 **
Piper betel Leaf Extract	200	43.72 ± 1.28*
Piper betel Leaf Extract	400	28.36 ± 1.04*

Table 8: Time taken for clot lysis

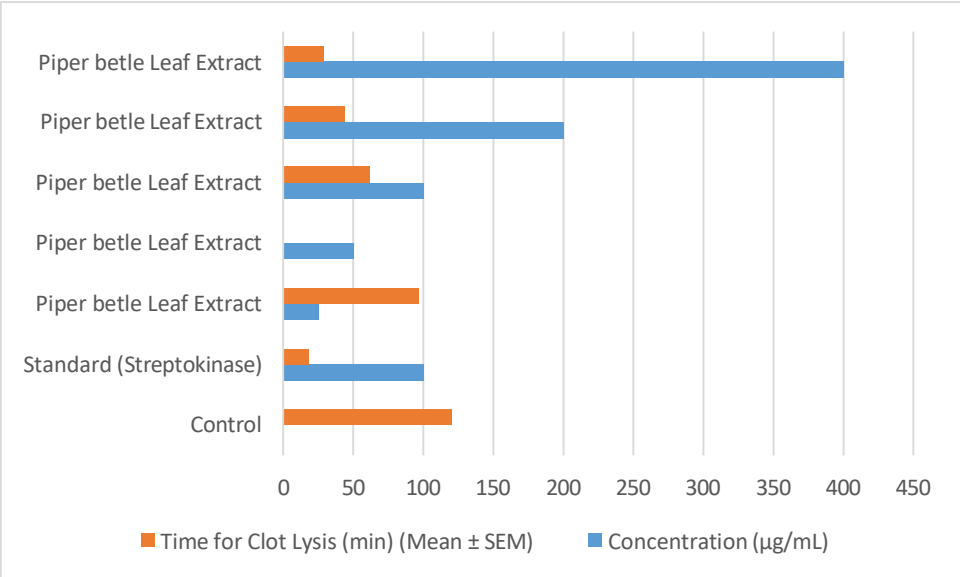


Figure 7 : Time taken for clot lysis[MIN]

Clot Weight Reduction Assay:

Treatment	Concentration (µg/mL)	Initial Clot Weight (g)	Final Clot Weight (g)	Clot Weight Reduction (%)
Control	—	0.524 ± 0.011	0.522 ± 0.010	0.38 ± 0.12
Standard (Streptokinase)	100	0.526 ± 0.010	0.138 ± 0.006*	73.76 ± 1.14*
Piper betel Leaf Extract	25	0.523 ± 0.012	0.436 ± 0.009	16.63 ± 0.95
Piper betel Leaf Extract	50	0.525 ± 0.011	*0.373 ± 0.008 **	*28.95 ± 1.02 **
Piper betel Leaf Extract	100	0.524 ± 0.010	0.303 ± 0.007 **	42.18 ± 1.07 **
Piper betel Leaf Extract	200	0.527 ± 0.011	0.231 ± 0.006*	56.17 ± 0.96*
Piper betel Leaf Extract	400	0.525 ± 0.010	0.172 ± 0.005*	67.24 ± 0.88*

Table 8: Clot weight reduction assay

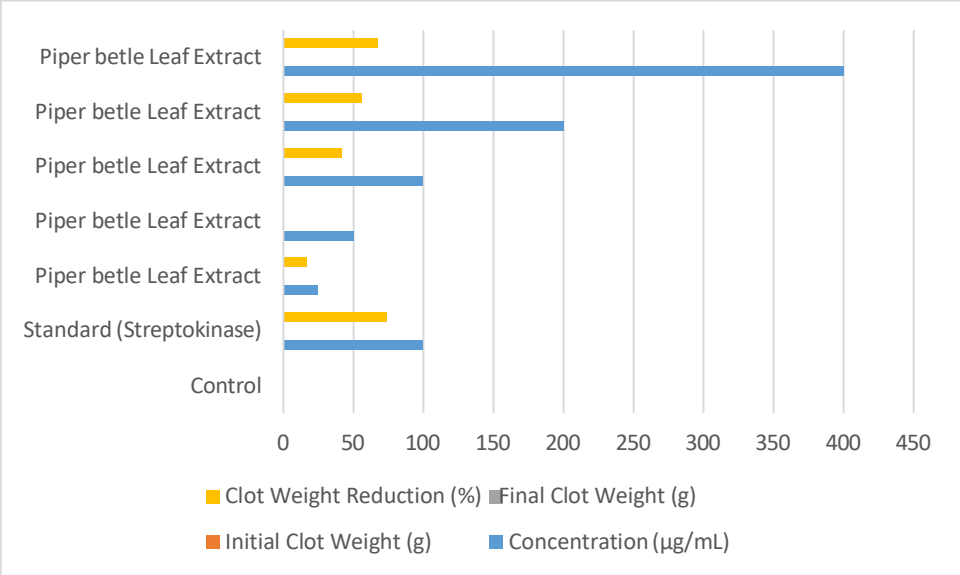


Figure 8: Clot weight reduction

DISCUSSION:

The present study was designed to investigate the in vitro and in vivo anti-urolithiatic aswell as thrombolytic activities of Piper betel leaf extract using well-established experimental models, and the results obtained clearly demonstrate the significant pharmacological potential. In the ethylene glycol-induced urolithiasis model, the disease control group showed marked elevation in urinary calcium and oxalate levels along with increased serum creatinine and kidney weight, indicating successful induction of renal stone formation and associated renal dysfunction. These changes are typically linked with hyperoxaluria, supersaturation of urinary solutes, and deposition of calcium oxalate crystals within renal tissues. Treatment with the standard drug Cystone significantly restored altered biochemical parameters toward normal levels, validating the experimental model. Importantly, Piper betel leaf extract produced a dose-dependent reduction in urinary and serum markers, suggesting its ability to reduce supersaturation of lithogenic substances, inhibit crystal deposition, and improve renal function. The in vitro anti-urolithiatic studies further supported these findings, where the extract significantly inhibited calcium oxalate crystallization and crystal growth in a concentration-dependent manner, indicating interference with crystal nucleation, aggregation, and growth processes. In the in vivo thrombolytic study using the $FeCl_3$-induced thrombosis model, the disease control group exhibited a significant increase in thrombus weight along with a reduction in platelet count, confirming successful induction of vascular thrombosis due to endothelial injury and platelet aggregation. Treatment with the standard drug Streptokinase demonstrated potent thrombolytic activity by markedly reducing thrombus weight and restoring platelet levels. Similarly, Piper betel leaf extract showed a dose-dependent reduction in thrombus weight and improvement in platelet count, indicating its ability to reduce clot burden and prevent platelet consumption. The in vitro clot lysis assay also revealed significant enhancement in percentage clot lysis and reduction in clot dissolution time with increasing concentrations of the extract, further confirming its fibrinolytic potential.The observed dual pharmacological activities may be attributed to the presence of bioactive phytoconstituents such as flavonoids, phenolic compounds, tannins, and alkaloids, which are known to possess strong antioxidant, anti-inflammatory, and membrane-stabilizing properties. These compounds may collectively contribute to inhibition of oxidative stress, prevention of endothelial damage, suppression of crystal formation, and modulation of coagulation pathways. Overall, the findings of this study strongly suggest that Piper betel leaf extract exhibits significant anti-urolithiatic and thrombolytic activities, thereby scientifically supporting its traditional medicinal use and highlighting its potential for further development as a natural therapeutic agent.

CONCLUSION:

The present study demonstrated that *Piper betel* leaf extract possesses significant anti-urolithiatic and thrombolytic activities in both in vitro and in vivo experimental models. In the ethylene glycol-induced urolithiasis model, the extract effectively reduced urinary calcium and oxalate levels, serum creatinine, and kidney weight, indicating a protective effect against calcium oxalate stone formation and improvement in renal function. The in vitro studies further confirmed its ability to inhibit calcium oxalate crystallization and crystal growth in a concentration-dependent manner, suggesting interference with key stages of urolithiasis development such as nucleation, aggregation, and crystal enlargement. In the $FeCl_3$-induced thrombosis model, *Piper betel*

extract significantly reduced thrombus weight and improved platelet count, indicating a clear thrombolytic potential. The in vitro clot lysis assay also supported these findings by showing increased percentage clot lysis and reduced clot dissolution time in a dose-dependent manner. The overall pharmacological effects may be attributed to the presence of bioactive constituents such as flavonoids, phenolic compounds, tannins, and alkaloids, which exhibit antioxidant, anti-inflammatory, membrane-stabilizing, and fibrinolytic properties.

In conclusion, *Piper betel* leaf extract exhibits promising dual therapeutic potential as an anti-urolithiatic and thrombolytic agent, thereby scientifically validating its traditional medicinal use. However, further studies involving isolation of active compounds, detailed mechanism of action, and clinical evaluation are recommended to establish its safety and efficacy for therapeutic applications.

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