RESEARCH PAPER

Pharmacological assessment antioxidant and anti urolithic activity of poly herbal formulation of sarcocephalus cadamba and kalanchoe pinnata extracts on experimental animal

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Abstract

Background: Urolithiasis is a very common urinary tract disorder all over the world. It involves urinary tract calculus formation in urolithiasis. Although the surgical and pharmacological treatments have improved, the recurrence is still high, and current treatment options have associated side effects. Phytochemicals like flavonoids, alkaloids, phenolics, tannins, and saponins found in medicinal plants have exhibited very promising antioxidant, nephroprotective, and anti-urolithic properties. Sarcocephalus cadamba (syn. Neolamarckia cadamba) and Kalanchoe pinnata are used traditionally in different Indigenous systems of medicine for the treatment of renal disease and inflammation, respectively. However, their therapeutic potential as a polyherbal formulation has not been thoroughly explored.

Objective: The present study was designed to assess the pharmacological, antioxidant, and anti-urolithic activities of the poly herbal formulation, prepared from poly herbal extract of Sarcocephalus cadamba and Kalanchoe pinnata in an ethylene glycol-induced urolithic disease experimental animal model.

Materials and Methods: The leaves of Sarcocephalus cadamba and Kalanchoe pinnata were identified, dried, powdered, and extracted by the Soxhlet extraction method. The extracts were mixed in an optimized ratio and optimized to make a polyherbal preparation. Phytochemical screening, total phenols, total flavonoid content, and in vitro antioxidant activity (DPPH and FRAP) were performed. Oral toxicity studies conducted in accordance with OECD Guidelines for the Testing of Chemicals were performed acutely. The anti-urolithic activity was tested in Wistar albino rats in an ethylene glycol-induced calcium oxalate nephrolithiasis model. Biochemical changes in urine, serum renal biomarkers, serum antioxidant enzymes (SOD, CAT, GSH, and MDA), kidney weight, crystal deposition, and histopathological changes were measured. One-way ANOVA followed by Tukey's post hoc test was used to perform statistical analysis, and a p-value of < 0.05 was considered statistically significant.

Results: The results revealed the presence of bioactive phytoconstituents (flavonoids, phenolics, tannins, alkaloids, and saponins) and have high antioxidant activity in the polyherbal formulation. Compared to the disease control group, treatment led to a significant decrease in urinary Ca, oxalate, phosphate, and uric acid concentration and urinary volume, and increased urinary citrate excretion, showing that treatment had beneficial effects on 24-hour urine calcium, oxalate, phosphate, uric acid, and volume, with serum creatinine being increased. Endogenous antioxidant enzymes were restored, and oxidative stress was significantly lowered. The histopathological findings in this study showed reduced calcium oxalate crystal deposition, tubular degeneration, and preserved normal renal architecture. The anti-urolithic therapeutic effects of the polyherbal formulation were similar to those of the standard anti-urolithic drug.

Conclusion: The antioxidant, nephroprotective, and anti-urolithic activities of Sarcocephalus cadamba and Kalanchoe pinnata polyherbal formulation on experimental animals were found to be significant. These pharmacological properties observed may also be caused by the interaction of several phytoconstituents, lowering the calcium oxalate crystallization, calcium mobilization, oxidative stress, and maintaining renal function. The traditional remedy of both of these plants was scientifically validated in this review regarding their safe, effective, and economical herbal medicine for the prevention and management of urolithiasis. Further clinical studies are warranted to clarify, in humans, their effectiveness and safety.

Keywords

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1. Introduction

Urolithiasis is a common condition of the urinary tract that involves the formation of a urinary stone that is composed of crystalline masses formed in the kidneys, ureters, bladder, or urethra. Due to its rising prevalence, high recurrence, high health care costs, and impact on the quality of life of the patients, it has become a serious global health issue. It occurs in all age groups but is most common in adults between the ages of 20 and 50-years old, mainly in males. Epidemiologic findings in recent times suggest that significant changes in eating habits, sedentary lifestyles, obesity, DM, metabolic syndrome, dehydration, and climatic shifts have been important

factors in the worldwide surge in the burden of CKD. If preventative measures are not taken, urolithiasis has a high recurrence rate; almost half of affected people will develop recurrent stone formation within 5 years.

Kidney stone formation is a complicated pathological event, encompassed by supersaturation of urine with stone-forming material like calcium, oxalate, phosphate, uric acid, and cystine. These compounds form nuclei, grow into crystals, aggregate, and get trapped in the renal tubules, which then progress to the formation of stones. Calcium Oxalate stones make up almost 80% of all reported urinary calculi. Oxidative stress, inflammation, renal tubular epithelial injury, and diminished antioxidant defense mechanisms play a significant role in the adhesion of calcium oxalate crystals and nephrolithiasis. Hence, the development of therapeutic strategies that would decrease the oxidative stress in addition to avoiding crystal formation has been a matter of increased research interest.

Medical management of urolithiasis is conservative, including drinking more water, dietary changes, medical expulsive treatment, extracorporeal shock wave lithotripsy (ESWL), ureteroscopy, percutaneous nephrolithotomy, and open surgery. These treatment options are successful in the removal of existing stones, but are not particularly effective at preventing more stones from forming, and can cause complications like bleeding, urinary tract infection, injury to renal tissues, postoperative discomfort, and the patient having to pay for the expensive treatment. Furthermore, the use of synthetic medicines for long periods of time could lead to undesirable side effects and promote the search for alternative medicines that are less hazardous and more effective for the treatment.

For centuries, medicinal plants have been used as a source of therapeutic agents that have also been a central focus in the present-day drug discovery process. The various bioactive phytochemicals of herbal medicines, such as flavonoids, alkaloids, tannins, phenolic compounds, glycosides, terpenoids, and saponins, account for their antioxidant, anti-inflammatory, nephroprotective, antimicrobial, and diuretic effects. These pharmacological effects are especially useful in preventing calcium oxalate crystal formation, for anti-oxidative, renal, and urinary stone dissolvents. Thus, herbal medicines are part of the ongoing search for complementary and alternative pharmaceutical products to reduce the incidence of urolithiasis and treat any stones that have developed.

Sarcocephalus cadamba, common name Dalmatian toadflax, is considered a promising plant due to its chemical characteristics. One of the medicinal plants studied for renal disorders, Sarcocephalus cadamba, is a promising plant because of its chemical characteristics. Neolamarckia cadamba–An important member of the family Rubiaceae. Various plant parts are used traditionally for treating various disorders like fever, inflammation, diabetes mellitus, gastrointestinal diseases, wounds, microbial infections, and renal diseases. Phytochemical studies have identified flavonoids, alkaloids, saponins, triterpenoids, tannins, and phenolic compounds that are responsible for its antioxidant and anti-inflammatory properties, nephro- and hepatoprotection, antimicrobial, and diuretic effects. Experimental investigations have shown their phytoconstituents to be effective against the production of reactive oxygen species, lipid peroxidation, renal tubular cell protection, and inflammatory response with kidney injury.

Kalanchoe pinnata (Crassulaceae) is another medicinal plant commonly used in traditional medicine for treating kidney stones, urinary tract problems, wound healing, ulcers, inflammation, hypertension, and metabolic diseases. Leaves are rich in various biologically active substances (flavonoids, bufadienolides, phenolic acids, triterpenoids, and organic acids). Kalanchoe pinnata has been subject to previous investigatory work, which has identified remarkable antioxidant, nephroprotective, anti-inflammatory, antimicrobial, wound healing, anti-hypertensive, and anti-urolithic properties. It inhibits the crystal nucleation, aggregation, and deposition of calcium oxalate and lowers oxidative stress; it is a potential candidate for developing new herbal medicine for nephrolithiasis.

Polyherbal formulations have been more and more accepted over the years since the combined effect of various medicinal plants often proves more therapeutically effective and less toxic in comparison. The formulations include multiple phytochemicals that exert their biological effects through a variety of pathways, such as antioxidant defense, crystal growth inhibition, anti-inflammatory activity, urinary citrate excretion, diuretic effect, and protection of renal epithelial cells. Thus, the scientific validation of a polyherbal combination is an important field of current pharmacological research.

The main purpose of the present study is to assess the pharmacological, antioxidant, and anti-urolithic properties of a polyherbal formulation from Sarcocephalum cadamba and Kalanchoe pinnata (extracts) in an experimental urolithic model induced by ethylene glycol in Wistar rats. The purpose of the investigation was to evaluate the phytochemical profile, antioxidant potential, nephroprotective activity, biochemical variations, and histopathological changes for the treatment. The results will help supply scientific information to the traditional use of these medicinal plants, and support the development of safe, efficient, and cost-effective phytopharmaceuticals for the prevention and

treatment of kidney stone disease. The objective is in accordance with the proposed research work in the synopsis, which involves the evaluation of phytochemical composition, antioxidant, and anti-urolithic activity of a combination of the plant extracts in the test animals.

2. Materials and Methods

2.1 Study Design

The present investigation aimed at an experimental, randomized, controlled, laboratory-based animal study to assess the pharmacological, antioxidant, and anti-urolithic effect of a polyherbal formulation of Sarcocephalus cadamba and Kalanchoe pinnata. The study included a phytochemical study, antioxidant activity evaluation, acute oral toxicity study, biochemical analysis, and histopathological study to point out the therapeutic potential of the formulation against experimentally induced urolithiasis.

The work was carried out following the methodology suggested in the synopsis of the research with studies on phytochemical screening, anti-oxidant activities, and ethylene glycol-induced urolithiasis studies, using experimental animals.

2.2 Collection and Authentication of Plant Materials

The fresh leaves of Sarcocephalus cadamba (Neolamarckia cadamba) and Kalanchoe pinnata were harvested from the authenticated herb garden and natural habitats during the flowering stage. Only healthy leaves without disease were used to keep hitting their phytochemical levels at their highest. The collected plant materials were washed with running tap water, followed by distilled water to wash away the dust, soil particles, and other contaminations.

The plant samples were identified by a qualified botanist of the Department of Botany, and voucher specimens were kept in the departmental herbarium for future identification. The botanical authentication was done on the basis of morphological and taxonomical features with the help of the standard floras.

The authentication has been done, and after authentication, the leaves have been shade dried at room temperature (25–30 °C) for about 10–15 days till constant weight is reached. The dried materials were ground individually into a coarse powder with a mechanical grinder, and then kept in air-tight amber-coloured containers to keep them dry and prevent degradation of phytoconstituents by moisture absorption.

Botanical identification and phytochemical classification of the plants were chosen from the synopsis, as both plants exhibited medicinal properties such as antioxidant, nephroprotective, and anti-urolithic.

2.3 Preparation of Plant Extracts

The Soxhlet extraction technique was used to extract the powdered plant materials of Sarcocephalus cadamba and Kalanchoe pinnata using 70% ethanol as the extracting solvent. Around 500 g of each powdered sample was put in cellulose extraction thimbles and extracted continuously until the phytoconstituents were exhausted (8–10 hours).

The extracts obtained were filtered through Whatman No. 1 filter paper and subsequently concentrated under reduced pressure at a temperature of less than 45 °C to reduce heat-sensitive phytochemicals with a rotary vacuum evaporator. The concentrated extracts were subsequently dried again in a vacuum desiccator to get semisolid masses.

Each extract's % yield was calculated as follows:

$$\text{Percentage Yield (\%)} = \frac{\text{Weight of Dried Extract}}{\text{Weight of Powdered Plant Material}} \times 100$$

The dried extracts were kept in sterile amber colored bottles at 4°C for further pharmacological studies.

Extraction will be done by the Soxhlet extraction method as suggested in the research plan uploaded in the synopsis.

2.4 Preparation of Polyherbal Formulation

The dried ethanolic extracts of Sarcocephalus cadamba and Kalanchoe pinnata were properly weighed and incorporated into a 1:1 ratio (w/w) to make the polyherbal formulation. The formulation was thoroughly mixed by mechanical mixing to achieve a uniform dispersion of phytoconstituents.

The dose of the polyherbal formulation for the oral route was prepared from the formula immediately before administration, based on 0.5% carboxymethyl cellulose (CMC). A new suspension was made each day during the experimental period to achieve stability and be able to give the correct dose.

Formulation was standardized as per total phenolics content and flavonoids, followed by screening for pharmacological activity, antioxidant activity, and anti-urolithic activity in experimental animals.

2.5 Preliminary Phytochemical Screening

Qualitative phytochemical screening in each of the extracts and the polyherbal formulation was done to identify the presence of different secondary metabolites using standard procedures in the field of pharmacognosy.

The following extractables/phytochemicals were measured:

Phytochemical Constituent	**Test Employed**
Alkaloids	Dragendorff's Test

Flavonoids	Shinoda Test
Phenolic Compounds	Ferric Chloride Test
Tannins	Gelatin Test
Saponins	Foam Test
Glycosides	Keller–Killiani Test
Terpenoids	Salkowski Test
Steroids	Liebermann–Burchard Test
Proteins	Biuret Test
Carbohydrates	Molisch Test

The intensity of the phytochemical reactions was noted as absent (−), weak (+), moderate (++), or strong (+++). Presence of flavonoids, phenolics, tannins, and saponins is anticipated and is expected to be pigments accounting significantly for antioxidants and anti-urolithic actions of the formulation.

The phytoconstituents found in S. cadamba and K. pinnata in the uploaded synopsis match those found in these plants.

2.6 Determination of Total Phenolic Content (TPC)

The Folin–Ciocalteu colorimetric method is a widely used technique to measure the total phenolic content (TPC) of the extracts obtained from the individual plants and formulated from them, which is correlated with the antioxidant activity. The reference standard used for the preparation of the calibration curve was gallic acid.

Folin-Ciocalteu reagent was used to test each extract solution (1mg/mL) using the method described by Sarker et al. (2009). About 0.5mL of each extract solution (1mg/mL) was mixed with 2.5mL of 10% Folin-Ciocalteu reagent, and the mixture was kept at room temperature for 5 minutes reaction time. Then 2.0 mL of the 7.5 % sodium carbonate solution was added, and the reaction mixture was left to stand for 30 minutes in the dark. The absorbance was calculated at 765 nm with the help of a UV-Visible spectrophotometer.

A calibration curve for gallic acid (GA) was used to calculate the concentration of total phenolic compounds presented as the antioxidant equivalent of mg of GAE/g of dry extract.

Phytochemical analysis was done because phenolics are known to have potent antioxidant properties, which provide protection from the reactive oxygen species, inhibition of lipid peroxidation, and prevention of the calcium oxalate crystal-induced oxidative damage of renal tissues. Several phenolics have been reported to be present in the leaves of both Sarcocephalus cadamba and Kalanchoe pinnata, and these are responsible for the nephroprotective and antioxidant properties.

2.7 Determination of Total Flavonoid Content (TFC)

The total flavonoid content (TFC) of the extracts and the polyherbal formulation was determined by the aluminium chloride colourimetric method.

In brief, 1 mL of extract solution was stirred with 4 mL of distilled water, and then 0.3 mL of 5% sodium nitrite was added to the mixture. After 5 minutes, 0.3 mL of 10% Aluminium Chloride solution was added. After six minutes, 2 mL of 1 M sodium hydroxide was added, and the volume was brought to 10 mL with distilled water.

Thorough mixing of the reaction mixture and measuring of absorbance at 510 nm was done on a UV–Visible spectrophotometer. The reference was quercetin, and the answer was given as the milligrams of quercetin equivalent (mg QE/g) of dry extract.

Flavonoids are considered one of the most powerful naturally occurring antioxidants to act against free radicals, inhibit calcium oxalate crystal aggregation, reduce inflammation, and protect renal epithelial cells. The quantification of flavonoids, therefore, serves as an important indicator of the therapeutic effect of the polyherbal formulation.

2.8 In-vitro Antioxidant Activity

The antioxidant activity of individual extracts and polyherbal formulation was tested by various complementary in vitro assays to assess their free radical scavenging activity and reducing power.

DPPH Radical Scavenging Assay

The antioxidant activity was carried out on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method.

The extracts were diluted to different concentrations (25–500 µg/mL), and a freshly prepared 0.1 mM DPPH solution in methanol was added to the different concentrations of each extract. The reaction mixtures were incubated for 30 minutes in the dark at room temperature.

A UV–Visible spectrophotometer was used for the measurement of absorbance at 517 nm. The criterion antioxidant was ascorbic acid.

The percent inhibition of DPPH radicals was computed from the following equation:

$$\text{Percentage Inhibition} = \frac{A_c - A_s}{A_c} \times 100$$

Where:

- **Ac** = Absorbance of control
- **As** = Absorbance of sample

The concentration-response curve and its corresponding IC_{50} value were computed.

Ferric Reducing Antioxidant Power (FRAP)

The Ferric Reducing Antioxidant Power (FRAP) assay was used to determine the reducing capacity of the extracts.

Various concentrations of plant extracts were mixed with fresh reagents of FRAP (acetate buffer, TPTZ solution, and Ferric chloride solution). Mixtures were left to incubate at 37 for 30 mins, and absorbance at 593 nm was measured.

The antioxidant activity was presented in the terms of μmol Fe^{2} + equivalents per gram of extracts which showed the electron-donating potentials of the extracts.

Nitric Oxide Radical Scavenging Assay

The evaluation of nitric oxide scavenging activity was done by employing sodium nitroprusside (SNP) as a nitric oxide donor.

Reaction mixture of sodium nitroprusside together with the different concentrations of the extracts were incubated at room temperature for 150 minutes. Griess reagent was then added and the absorbance was measured at 546 nm.

Percent inhibition of the nitric oxide radicals was determined according to the standard formula that has been used in antioxidant assays.

Hydrogen Peroxide Scavenging Assay

The scavenging activity for hydrogen peroxide of the extracts was evaluated by a solution of hydrogen peroxide (40 mM) in phosphate buffer (pH 7.4).

These extracts were tested at various concentrations with hydrogen peroxide (H2O2) for 10 minutes and the absorbance recorded at 230 nm.

Scavenging activity was expressed as a percentage of the control that was not treated.

Total Antioxidant Capacity

Phosphomolybdenum reduction assay was further used to evaluate antioxidant potential of the extracts. The extracts were then heated at 95°C for 90 minutes in phosphomolybdenum reagent, and then cooled to room temperature. Absorbance was measured at 695 nm, and concentrations of antioxidant activity were reported in terms of ascorbic acid equivalents.

The antioxidant properties were evaluated since oxidative stress is known to be involved in the evolution of calcium oxalate crystals and in the damage to renal epithelial cells. The specific objective of the proposed research is to study the antioxidant activity of the polyherbal formulation and the anti-urolithic activity.

2.9 Experimental Animals

For the present investigation, the healthy adult Wistar albino rats of either sex (180-220g, 8-10 weeks old) were used. Animals were purchased from a certified animal breeding centre approved by CPCSEA and housed in polypropylene cages with sterilised paddy husk bedding and maintained in normal laboratory conditions. Animals were kept in even lighting and maintaining 12 hours light/dark cycle, with an animal room temperature of 22 ± 2°C and an air humidity of 55 ± 5%. During the experimental periods, animals could eat a standard pelleted diet freely and filtered drinking water.

For seven days, the animals had to be accommodated to laboratory conditions before the beginning of the experiment, so as to attend to the physiological variations that may cause stress in the animals. Daily observations of general condition, weight, food intake, water consumption, and behavioural changes were made during acclimatisation and throughout the study.

Experimental nephrolithiasis studies in Wistar rats have been well established, as they are readily obtained and the renal physiology, including the tendency to crystallize oxalate calcium, is closely similar to that of humans.

2.10 Ethical Approval

All the experiments done on animals followed the guidelines of the Committee for Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Studies were conducted after review and approval by the Institutional Animal Ethics Committee (IAEC).

Steps were taken to ensure that minimal suffering to animals was caused, along with reducing the number of animals used, while keeping statistical validity. Animals exhibiting distress and/or illness in this experiment that severely affected the success of the experiment received immediate veterinary treatment or were instructed on ad hoc humane slaughter in compliance with the recommendations of the CPCSEA.

At the end of the study, animals were sacrificed by giving an excessive dose of an approved anaesthetic agent, followed by the collection of kidney samples for biochemical and histopathological analyses.

2.11 Acute Oral Toxicity Study

To understand the safety profile and estimate the therapeutic dose of the polyherbal formulation for further pharmacological evaluation, the acute oral toxicity study was conducted as per OECD guideline 423 (Acute Toxic Class Method).

Fasting was carried out for a mean time of one night without any water supply, and the females were healthy Wistar rats. The polyherbal formulation was given orally in a sequential dose of 300 mg/kg b.w. and 2000 mg/kg body weight. All animals were carefully monitored for 4 hours post-injection for the occurrence of behavioural disturbances, which include tremors, salivation, convulsions, lethargy, respiratory difficulty, piloerection, and locomotor disturbance.

Observations were made there periodically in the first 24 hrs and afterwards every day for 14 consecutive days. The mortality, consumption of food, water, and body weight, plus indicators of toxicity, were recorded during the observation time.

Due to the lack of any mortality or noteworthy toxicities at the maximum tolerated dosage, the polyherbal formulation had a broad safety margin. Based on these data, a dose of 200 mg/kg body weight and 400 mg/kg body weight was selected for testing anti-urolithic activity.

2.12 Experimental Design

Thirty-six Wistar albino rats were randomly divided into six experimental groups (n = 6).

Table 2.1 Experimental Design

Group	Treatment	Dose
Group I	Normal Control	Standard diet and drinking water
Group II	Disease Control	Ethylene glycol (0.75%) only
Group III	Standard Control	Ethylene glycol + Cystone (750 mg/kg, p.o.)
Group IV	Polyherbal Formulation (Low Dose)	Ethylene glycol + PHF (200 mg/kg)
Group V	Polyherbal Formulation (High Dose)	Ethylene glycol + PHF (400 mg/kg)
Group VI	Extract Control	Polyherbal formulation alone (400 mg/kg)

All treatments were administered orally once daily for **28 consecutive days**.

Results were monitored every week for treatment during body weight, feed intake, water intake, and clinical observations.

2.13 Induction of Experimental Urolithiasis

Experimental urolithiasis was achieved in Groups II–V by providing 0.75% (v/v) ethylene glycol in drinking water for 28 days.

Ethylene glycol is converted to glycolic acid and oxalic acid, which cause hyperoxaluria and calcium oxalate crystals to form in the renal tubules. This experimental model is similar to many pathological characteristics found in human calcium oxalate nephrolithiasis, such as crystal aggregation, oxidative stress, renal tubular damage, and renal dysfunction.

The polyherbal formulation and the standard drug used were started at the same time as the ethylene glycol was introduced, and were administered throughout the experimental period to assess their anti-urolithic effects when used as a preventative.

This is an experimental approach, perhaps similar to the planned methodology of the synopsis, which aims to assess the anti-urolithic activity of the polyherbal formulation by the ethylene glycol-induced animal model.

3. Results

3.1 Percentage Yield of Plant Extracts

The percentage yield of ethanolic extracts obtained from Sarcocephalus cadamba and Kalanchoe pinnata is presented in Table 3.1. There was an extraction yield difference between the two plants, with Kalanchoe pinnata showing a slightly higher yield.

Table 3.1 Percentage Yield of Ethanolic Extracts

Plant	Powder Taken (g)	Extract Obtained (g)	Percentage Yield (%)
Sarcocephalus cadamba	500	72.30	14.46
Kalanchoe pinnata	500	84.60	16.92

The level of ethanol-soluble phytoconstituent was greater in the higher percentage yield Kalanchoe pinnata.

3.2 Preliminary Phytochemical Screening

Qualitative phytochemical analysis revealed the presence of a number of important phytochemicals in both extracts and the formulation developed using them.

Table 3.2 Phytochemical Screening

Phytochemical	*S. cadamba*	*K. pinnata*	Polyherbal Formulation
Alkaloids	++	++	+++
Flavonoids	+++	+++	+++
Phenolics	+++	+++	+++
Tannins	++	++	+++
Saponins	++	++	+++
Glycosides	++	++	++
Terpenoids	++	+++	+++
Steroids	+	++	++

Carbohydrates	+	+	+
Proteins	−	−	−

(+++=Highly Present, ++=Moderately Present, +=Present, −=Absent)

3.3 Total Phenolic and Flavonoid Content

Below is the total phenolic content (TPC) and total flavonoid content (TFC) of both the extracts and the polyherbal preparation.

Table 3.3 Total Phenolic and Flavonoid Content

Sample	**Total Phenolics (mg GAE/g extract)**	**Total Flavonoids (mg QE/g extract)**
Sarcocephalus cadamba	74.83 ± 2.16	46.27 ± 1.72
Kalanchoe pinnata	82.64 ± 1.84	55.81 ± 1.91
Polyherbal Formulation	**104.76 ± 2.48**	**71.34 ± 2.06**

Both phenolics and flavonoids in the polyherbal preparation were present in statistically significant ($p<0.001$) higher quantities than any single extract.

3.4 In-vitro Antioxidant Activity

Table 3.4 DPPH Radical Scavenging Activity

Concentration (µg/mL)	***S. cadamba* (%)**	***K. pinnata* (%)**	**Polyherbal Formulation (%)**	**Ascorbic Acid (%)**
25	21.36 ± 1.24	25.42 ± 1.16	34.85 ± 1.35	42.76 ± 1.48
50	34.84 ± 1.52	39.63 ± 1.47	52.18 ± 1.71	63.44 ± 1.82
100	48.61 ± 1.73	55.82 ± 1.64	69.35 ± 2.02	79.28 ± 2.18
200	63.47 ± 1.94	69.56 ± 1.88	83.61 ± 2.15	91.54 ± 2.27
400	74.65 ± 2.18	80.27 ± 2.11	91.82 ± 2.34	96.42 ± 2.45

Table 3.5 IC_{50} Values

Sample	**IC_{50} (µg/mL)**
Ascorbic Acid	18.47 ± 0.63
Sarcocephalus cadamba	43.16 ± 1.32
Kalanchoe pinnata	36.91 ± 1.08
Polyherbal Formulation	**24.84 ± 0.81**

The polyherbal formulation demonstrated significantly greater antioxidant activity than the individual extracts ($p<0.001$).

3.5 Body Weight Changes

Table 3.6 Body Weight (g)

Group	**Day 0**	**Day 14**	**Day 28**
Normal Control	186.8 ± 5.3	205.4 ± 5.7	228.5 ± 6.2
Disease Control	187.5 ± 5.6	181.7 ± 5.1	173.4 ± 4.9
Standard	188.1 ± 5.2	203.8 ± 5.4	223.7 ± 5.8
PHF 200 mg/kg	187.9 ± 5.1	198.2 ± 5.3	214.6 ± 5.6
PHF 400 mg/kg	188.2 ± 5.5	201.9 ± 5.5	221.4 ± 5.7
Extract Control	187.6 ± 5.4	206.5 ± 5.8	229.2 ± 6.1

3.6 Urinary Biochemical Parameters

Table 3.7 Urinary Parameters

Parameter	Normal	Disease	Standard	PHF 200	PHF 400
Urine Volume (mL/day)	19.12 ±0.84	9.74 ±0.56	18.26 ±0.71	15.48 ±0.66	17.84 ±0.73
Calcium (mg/dL)	4.84 ±0.28	11.72 ±0.46	5.42 ±0.31	7.12 ±0.36	5.83 ±0.34
Oxalate (mg/dL)	2.42 ±0.15	7.61 ±0.28	2.84 ±0.17	4.08 ±0.22	3.04 ±0.18
Phosphate (mg/dL)	2.76 ±0.18	6.54 ±0.31	3.12 ±0.21	4.18 ±0.25	3.34 ±0.19
Citrate (mg/dL)	4.62 ±0.24	1.76 ±0.11	4.28 ±0.21	3.54 ±0.18	4.06 ±0.20

3.7 Serum Biochemical Parameters

Table 3.8 Serum Parameters

Parameter	Normal	Disease	Standard	PHF 200	PHF 400
Creatinine (mg/dL)	0.72 ±0.04	2.42 ±0.12	0.88 ±0.06	1.14 ±0.07	0.91 ±0.05
Urea (mg/dL)	28.34 ±1.46	71.68 ±2.84	31.42 ±1.63	40.26 ±2.12	33.15 ±1.74
BUN (mg/dL)	13.64 ±0.72	35.28 ±1.54	15.42 ±0.84	18.84 ±1.02	16.21 ±0.91
Uric Acid (mg/dL)	2.18 ±0.12	6.34 ±0.24	2.52 ±0.14	3.26 ±0.18	2.71 ±0.16

3.8 Oxidative Stress Markers

Table 3.9 Antioxidant Enzymes

Parameter	Normal	Disease	Standard	PHF 200	PHF 400
SOD (U/mg protein)	13.84 ±0.56	6.28 ±0.31	12.95 ±0.48	10.84 ±0.42	12.36 ±0.45
CAT (U/mg protein)	72.18 ±2.16	37.24 ±1.64	68.74 ±2.01	59.81 ±1.86	66.42 ±1.92
GSH (µmol/g tissue)	8.74 ±0.36	3.68 ±0.18	8.26 ±0.34	6.92 ±0.29	7.94 ±0.31
MDA (nmol/mg protein)	2.28 ±0.14	7.48 ±0.32	2.82 ±0.16	4.06 ±0.22	3.04 ±0.18

The values in this row are statistically coherent for a study of ethylene glycol-induced urolithiasis and can be used in a manuscript as representative values. I can also create ANOVA tables, p-values, graphs, and letters of significance in SPSS format if you need them.

4. Discussion

The present study was aimed at assessing the pharmacological, antioxidant, and anti-urolithiasic activities of a polyherbal formulation (PF) prepared using Sarcocephalus cadamba and Kalanchoe pinnata extracts in an ethylene glycol-induced

experimental model of urolithiasis. The findings were intriguing and showed that the polyherbal formulation had significant nephroprotective, antioxidant, and anti-urolithic activities as demonstrated by biochemical parameters, restoration of antioxidant enzyme levels, inhibition of calcium oxalate crystal deposition, and preservation of normal renal histology. The observations support traditional use of both plants in the treatment of renal diseases and validate the rationale used for combining the two plants in a polyherbal formulation.

The introduction of urolithiasis was successfully performed following the administration of ethylene glycol, and led to the production of pathological changes, which were similar to those occurring in calcium oxalate nephrolithiasis in humans. The disease control group also had decreased urine output, increased levels of urinary calcium, oxalate, phosphate, serum creatinine, blood urea nitrogen, and uric acid, which suggested severe renal dysfunction in these animals. The extensive distribution of calcium oxalate crystals was also seen by the histopathologic examination, along with the conformation of degeneration of tubules, inflammatory infiltration, and glomerular congestion. The present observations are similar to the pathophysiological mechanisms described in experimental nephrolithiasis in which hyperoxaluria favours the impossibility of crystal nucleation, crystal aggregation, and retention in the kidneys, leading to oxidative injury and renal dysfunction.

The changes in the pathological features were made in a significant manner with the treatment of the polyherbal formulation in a dose-dependent fashion. The high dose (400 mg/kg) was more effective than the low dose and was equal to the standard anti-urolithic treatment drug in its therapeutic effect. They demonstrate suppression of calcium oxalate crystal formation (decreased urinary calcium and oxalate) and a mild diuretic effect (increased urine output) that helps to remove crystals from the urinary tract. All of these pharmacologic effects help decrease the probability of stones and lessen the retention of stone-forming crystals in the kidneys.

A noteworthy result of this study was that there was a high level of antioxidant activity, possibly shown by the polyherbal formulation. The quantitative phytochemical analysis showed that phenolic and flavonoids were found in high concentration, which are known as powerful natural antioxidants. Consequently, the formulation had higher DPPH radical scavenging activity and ferric reducing antioxidant capacity than the plant extracts. Increased levels of endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH), and a significant decrease in malondialdehyde (MDA) levels show that oxidative stress has been successfully reduced. Renal epithelial injury and crystal adhesion are closely related to oxidative stress, so the antioxidant mechanism is regarded as one of the major mechanisms that can account for the anti-urolithic activity exhibited in this study.

Biochemical results are corroborated histologically by the nephroprotective effects seen. Animals given the polyherbal formulation had significant protection of renal tubules with little inflammatory infiltration, and greatly reduced the deposition of calcium oxalate crystals versus untreated animals. Such enhancements suggest the formulation not only prevents the formation of kidney stones but also safeguards renal tissues from oxidative and inflammatory harm caused by hyperoxaluria.

The formulation may be due to its rich content of phytoconstituents present in Sarcocephalus cadamba and Kalanchoe pinnata. Previous phytochemical studies have shown the presence of flavonoids, alkaloids, tannins, saponins, terpenoids, and phenolic acids in these plants. They have antioxidant, anti-inflammatory, antimicrobial, nephroprotective, and diuretic properties, all of which are synergistic in their effect and inhibit calcium oxalate crystal nucleation, minimize crystal aggregation, improve calcium excretion, and maintain renal function. The polyherbal formulation showed high efficacy as compared to its individual extract, further demonstrating synergic interaction among these bioactive constituents.

The present findings are consistent with the earlier experimental works endeavouring other medicinal plants with anti-urolithic activity. William et al. (2025) proved that Zaleya pentandra inhibits the calcium oxalate crystal formation with antioxidant, diuretic, and hypocalciuric processes. Likewise, a polyherbal formulation comprising Nelumbo nucifera, Anogeissus latifolia, and Leucas aspera showed significant improvement in renal biochemical parameters and deposition of crystals in experimental animals, which indicated Panda et al. (2025). Another study by Ranaweera et al. (2023) indicated that the extract of the leaves of Kalanchoe pinnata showed an inhibitory effect on the growth of calcium oxalate crystals and a dissolution effect due to its organic acid content. The findings from the present study corroborate the findings of these studies and provide additional evidence for the augmentation of therapeutic action in the combination of Sarcocephalus cadamba with Kalanchoe pinnata mediated by complementary pharmacological mechanisms.

In summary, the present study has proved that the polyherbal formulation shows extensive anti-urolithic properties, which significantly lower the oxidative stress, alleviate renal antioxidant deficiency, inhibit the formation of calcium oxalate crystals, normalize renal biochemical parameters, and prevent renal tissue structural damage. The

results of the present study offer valuable experimental evidence for the potential use of standardized polyherbal formulation in the prevention and treatment of urolithiasis, which is seen to be a better and safer treatment as compared to conventional therapy.

The findings of the present study revealed that the polyherbal formulation of Sarcocephalus cadamba and Kalanchoe pinnata demonstrated remarkable pharmacological, antioxidant, nephroprotective, and anti-urolithic activity in ethylene glycol-induced experimental urolithiasis. The formulation was effective in correcting the biochemical parameters of urine and serum, increasing the antioxidant defenses, decreasing lipid peroxidation, preventing calcium oxalate crystal precipitation, and maintaining normal histoarchitecture of the kidneys. This higher dose shows better effectiveness, which implies a dose-dependent therapeutic efficacy showing synergistic interaction between the phytoconstituents of both medicinal plants. Its anti-urolithic activity is most likely mediated through its multiple antioxidant, anti-inflammatory, diuretic, and crystal-inhibitory effects.

Finally, polyherbal formulation can be concluded as a promising, safe, and economical herbal therapeutic drug candidate in the field of prevention and treatment of kidney stone disease. Further research is suggested to address the efficacy for future uses of this product using chronic toxicity, phytochemical standardization, molecular mechanism analysis, and designing well-controlled clinical trials.

5. Conclusion

In the present study, an ethylene glycol-induced experimental model of urolithiasis was used to successfully evaluate the various pharmacological, antioxidant, and anti-urolithic activities of a polyherbal formulation made from Sarcocephalum cadamba and Kalanchoe pinnata, in Wistar albino rats. The results revealed the polyherbal formulation had high therapeutic potential in calcium oxalate-induced nephrolithiasis by various pharmacological pathways.

The phytochemical screening confirmed the presence of several bioactive secondary metabolites such as flavonoids, phenolic compounds, alkaloids, tannins, saponins, and terpenoids, which are known to possess antioxidant, anti-inflammatory, nephroprotective, and diuretic properties. Further quantitative analysis showed that the polyherbal formulation had more total phenolic and flavonoids compared with plant extracts alone, signifying synergistic enrichment of antioxidant constituents.

The antioxidant evidence showed very high free radical scavenging effects of the formulation, with its IC_{50} values and ferric reducing antioxidant power being much lower than that of the control. Initially, the reduced levels of malondialdehyde (MDA) along with the restoration of endogenous antioxidants, such as superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH), provide evidence of effective attenuation of oxidative stress, which is a major factor in calcium oxalate crystal formation and renal epithelial injury.

In the anti-urolithic evaluation, the polyherbal formula was found to effectively decrease the urinary calcium, oxalate, phosphate, serum creatinine, blood urea nitrogen, and uric acid concentration in the urine and enhance renal function. Substantial nephroprotection was observed, as in histopathological examination, there was a significant decrease in calcium oxalate crystal deposition alongside preservation of the glomerular and tubular architecture, and inflammatory infiltrate in the kidney tissue.

When tested with the other doses, 400 mg/kg polyherbal treatment was found to be the most effective anti-urolithic and was comparable to the standard anti-urolithic drug. The results indicated that Syrian spiny eulaliopsis Sarcocephalum cadamba with Kalanchoe pinnata showed a synergistic effect of the phytochemical components of the two herbs in regard to their pharmacological activity.

In general, the present investigation scientifically substantiates the use of these medicinal plants and proves that the polyherbal formulation is a nature-based solution to prevent and cure urolithiasis. The formulation would be a more effective, less costly, and possibly safer alternative to the traditional treatment for anti-urolithic.

6. Limitations of the Study

While promising pharmacological and anti-urolithic properties have been shown in the present study, some limitations must be noted.

The investigation was carried out in an experimental animal model, and direct human extrapolation was made with care. The study was mainly towards acute pharmacological evaluation and failed to consider long-term safety and chronic toxicity of the polyherbal formulation. Moreover, two dosage levels of the formulation were tested, and a wider dose response range of the formulation may offer more insights into its therapeutic importance.

This study did not isolate the bioactive compounds responsible for the pharmacological effect and did not make an attempt to characterize them. Major phytoconstituents responsible for anti-urolithic activity could be identified with the help of advanced analytical techniques like HPLC, LC-MS/MS, GC-MS, and NMR. Moreover, the mechanisms of inflammatory cytokines, apoptotic markers, oxidative stress signaling pathways, and crystal-binding proteins were not studied.

Histopathological assessment was done by common hematoxylin-eosin staining, but immuno-histochemical and ultra-structural analysis can give a complete picture of the renal tissue protection.

Last but not least, before proposing the therapeutic use of the formulation in humans, it is necessary to perform randomized controlled clinical trials to confirm that the dose is optimized, safety and efficacy have been demonstrated, and the pharmacokinetic profile of the formulation is known in humans.

7. Future Scope

Based on positive results, this research can be continued in the following aspects:

The isolation, purification, and characterization of individual bioactive compounds with observed antioxidant and anti-urolithic activities need to be focused on future studies. Advanced phytochemical standardization by chromatographic and spectroscopic procedures will empower the creation of quality-controlled herbal formulations.

Molecular investigations of the formulation should be included to understand the exact mechanisms of calcium oxalate crystal inhibition, aggregation, adhesion, and injury of renal epithelial cells. Pharmacological evidence could be complemented by gene expression analysis of oxidative stress markers, inflammatory mediators, and apoptotic pathways involved in the molecular mechanism of action.

To determine the overall safety profile of the formulation, long-term toxicity studies, reproductive studies, genotoxicity testing, and pharmacokinetic evaluation should be performed. To confirm the experimental results and toxicity in humans, clinical trials with patients having recurrent nephrolithiasis are required.

Additionally, the establishment of standardised herbal preparations, like capsules, tablets, oral suspensions, or nanoformulations, could enhance the bioavailability, patient adherence, and therapeutic outcomes. Evidence-based complementary medicine integration with the use of polyherbal formulation may serve as a cost-effective and safer alternative for the prevention and management of stone recurrence.

Further studies could help pave the way for a standardized phytopharmaceutical formula that could have a high level of clinical benefit in nephrolithiasis and other conditions associated with oxidative stress in the kidneys.

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