Antiurolithiatic Activity of *Calliandra Haematocephala* Leaf Extract in Ethylene-Glycol Induced Wistar Rat Model

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ABSTRACT

Background

Traditionally, *Calliandra haematocephala* is used for the treatment of kidney stones. Urolithiasis, also known as the formation of urinary stones, is a health problem that affects nearly all populations worldwide, causing severe acute back pain and occasionally leading to more severe complications, such as pyelonephritis or acute renal failure. The antiurolithiatic activity of this plant has not been documented, hence this study sought to incorporate chemical analysis such as HPLC and GC-MS and biological evaluation to ascertain the effect of this plant in ethylene-glycol-induced Wistar rats. The presence of secondary metabolites was detected using standard methods. Agilent 1100 HPLC with a variable wavelength UV detector and Shimadzu GC-MS-QP2010 Plus were used for the HPLC and GC-MS analysis respectively. Antimicrobial analysis was done using the diffusion method, and a Membrane Perturbation assay was performed to evaluate the ability of the plant to stabilize the membrane. Biochemical and histological analysis was performed using commercial kits.

Results

The instrumental analysis showed the presence of gallic acid, quercetin, squalene (0.162 %), alpha-d-mannofuranoside (0.312%) and so on. *C. haematocephala* exhibited moderate inhibition against some bacteria strains selected for the study including *Escherichia coli* (10 mm) at 250 mg/mL. The plant also showed good potential in stabilizing the membrane. Significant differences ($p<0.05$) in the levels of the urea and creatinine were also observed. The nephroprotective effect of the plant further revealed normal histoarchitecture of the kidney in a dose-dependent manner.

Conclusion

The findings provide a robust foundation for the development of novel phytotherapeutics for managing and preventing urolithiasis.

Keywords: Urolithiasis, Histological parameters, Ethylene-glycol, Membrane Perturbation, Nephroprotective effect.

BACKGROUND

Urolithiasis, also referred to as urinary stone production, is a medical condition that impacts almost every population on the planet. In addition to causing excruciating back pain, it can sometimes result in more serious

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side effects such as acute renal failure or pyelonephritis. With a lifetime prevalence of roughly 10% for men and 6% for women, kidney stone formation is a common urological issue on the rise in many developed nations (Dirie *et al*., 2023; Stamatelou *et al.*, 2023). Within ten years following initial treatment, the recurrence rate is close to 60% (Ashok et al., 2010). Dietary changes and enough hydration intake may effectively stop stone recurrence. Treatments for various kinds of stones include tiopronin, potassium alkali, thiazide, and allopurinol. Urolithiasis, commonly referred to as urinary stones, has plagued humans since 4000 BC. Globally, there are wide variations in the prevalence of this illness. It is estimated that the prevalence of urolithiasis is 2-13% in low-income countries and 1-5% in high-income nations worldwide (Ather, 2022). Contrary to earlier studies that depicted urinary stone disease as being rare in Nigeria (E *et al.*, 2024), a number of recent reports have shown an increasing incidence. As one of the largest economies in Africa, Nigerians have moved from traditional agro-based African diets to the relatively additive-laden and processed western diet. There have also been suggestions of temperature increases during hot seasons. It has been suggested that the socioeconomic growth of Sub-Saharan countries and the changing climate are potential explanations (Daphnee *et al*., 2024). *Calliandra haematocephala* is a spreading shrub with many branches that can reach a height of one to three meters (Ashour *et al.*, 2023). It belongs to the Mimosaceae family. Numerous studies have demonstrated the anti-inflammatory, gastroprotective, anticonvulsant, immunomodulatory, and antiulcerogenic qualities of *Calliandra haematocephala*. This plant has long been used as an antibacterial agent, and research has shown that both the bark and the blooms of the plant contain antibacterial properties. According to one study, silver nanoparticles made from *Calliandra haematocephala* leaf extract had antibacterial efficacy against E. coli. *Calliandra haematocephala*, commonly referred to as red powder puff, is a member of the Fabaceae family (Ashour *et al.*, 2023). It is a decorative plant with an evergreen shrub that spreads widely and has many branches. According to studies, red powder puff contains immune-adjuvant, anti-inflammatory, anticonvulsant, antiulcerogenic, and gastroprotective qualities. The decoction of the flower extract is used as a blood purifier and tonic everywhere because of its antioxidant properties. Its roots are used in the relief of haemorrhoids (Siddhi *et al*., 2023). It is reported that betulinic acid in the plant is responsible for its antitumor, anti-HIV, and anti-rotaviral activity. It is also used for hepato-protection and has antioxidant activity, due to the free radical scavenging effect by the phenolics, saponins, and flavonoid contents of the extract (Erharuyi *et al*., 2022).

However, over the previous ten years, few new treatments have been developed, and treating some kinds of stones is still difficult. This study was motivated by the urgent need to evaluate the biology and aetiology of stone disease utilising medicinal plant extract. Therefore, the purpose of this study is to investigate the chemical component of this plant that may aid in the treatment of kidney stones in a Wistar rat that has been induced with ethylene glycol.

METHODS

Sample collection and preparation

The process of plant collection and preparation was done according to the method of Ajiboye *et al.*, (2020). The leaves of *Calliandra haematocephala* were gathered from the Adeyemi Federal University of Education, Ondo Garden. *Calliandra haematocephala* plants were authenticated at the Department of Biological Sciences, University of Medical Sciences, Ondo and specimen numbers, UNIMED/PBTH/0079 were generated for *Calliandra haematocephala*. The voucher specimen was kept in the herbarium unit for future use. The leaves of the plant were cleaned using distilled water, dried at room temperature in a ventilated space for two days, and then ground into a fine powder, labelled, kept in an airtight container and stored away from moisture until analysis.

- All the chemicals used were of analytical grade.

Extraction of the plant sample

- Extraction was performed using methanol and ethyl acetate.

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- The powdered plant was extracted by soaking in methanol and ethyl acetate separately for 3 days, after which it was filtered and concentrated using a rotary evaporator at ≤ 45 degrees Celcius and stored for further analysis.

Phytochemical Screening

- Separate milled samples were steeped in methanol and ethylacetate (50:50) at room temperature (25 ^{o}C) for 24 hours before being filtered and screened for phytochemicals. The standard approach was used for the phytochemical screening to detect natural compounds like flavonoids, tannins, etc.

Preliminary Phytochemical Screening of the Plants

- **Test for Flavonoids**: 2 g of the extract underwent a series of chemical tests. Initially, 20% sodium hydroxide was introduced dropwisely, resulting in the observation of a vivid and intense yellow colour formation. Subsequently, 70% dilute HCl was also introduced into the mixture, leading to the disappearance of the previously formed yellow colour. The formation and subsequent disappearance of the yellow colour served as a clear indicator of the presence of flavonoids in the extract.

- **Test for Alkaloids**: The following procedure was used to assess the presence of alkaloids in the sample extract: 1 g of each extract was combined with 1 ml of Marquis Reagent, along with 2 ml of concentrated sulfuric acid and a few drops of 40% formaldehyde. The emergence of a dark orange colour served as a clear indicator of the presence of alkaloids within the samples (Meihua *et al.,* 2022).

- **Test for Saponin:** The following procedure was used to determine whether the samples contained saponins: Two grams of each extract were mixed with six milliliters of distilled water and briskly shaken. Continuous foam production confirmed the presence of saponins.

- **Test for Tannins**: The presence of tannins in the samples was detected using the following approach. 2 g of each extract was immersed in a 10 % alcoholic ferric chloride solution. The appearance of a distinct brownish-green colour served as a reliable indicator of tannin content in the extracts.

- **Anthraquinones:** In the analytical process, 500 mg of dried plant leaves were subjected to boiling in 10 % HCl for a duration of 5 minutes. Afterward, the resulting filtrate was left to cool. To this 2 mL filtrate, an equal volume of $CHCl_3$ was introduced, along with a few drops of 10 % NH_3. The emergence of a distinct rose-pink colour in the mixture served as a conclusive indication of the presence of anthraquinones.

- **Steroids:** To investigate the presence of steroids within the plant extract (Mgamat *et al.,* 2023), the following procedure was performed: 1 gram of plant extract was mixed with an equal amount of chloroform. A few drops of concentrated sulfuric acid were added to the mixture, resulting in the creation of a prominent brown ring. This brown ring indicated the presence of steroids in the extract.

- **Test for Terpenoids**: A unique approach was used to examine terpenoids in the extract: 1 g of extract was mixed with 0.5 ml of chloroform, followed by a few drops of strong sulfuric acid. A reddish-brown precipitate was seen, indicating the presence of terpenoids in the extract.

- **Test for Glycosides**: To detect the presence of cardiac glycosides in the sample extract, a specific approach was employed: 1 g of each extraction was combined with 0.5 ml of glacial acetic acid and three drops of 1 % aqueous ferric chloride solution. A large brown ring produced at the contact site indicated the presence of cardiac glycosides in the extract.

Proximate Composition Analysis

Moisture Content

Moisture is determined by the loss in weight that occurs when a sample is dried to a constant weight in an oven. About 2 g of a sample was weighed into a silica dish previously dried and weighed. The sample was

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then dried in an oven at 65 ^{0}C for 36 hours, cooled in a desiccator, and weighed. The drying and weighing continued until a constant weight was achieved. The difference in weight was recorded as the moisture content while the percentage moisture content was estimated as follows:

Average weight loss: Sample A = $\frac{\text{Wt 1-Wt 2}}{2}$

$$\% \text{ Moisture Content} = \frac{\text{Sample A}}{2} \times \frac{100}{1}$$

Determination Of Ash Content

Ash content was determined by holding a clean flat-bottomed silica dish in a hot burner flame for 1 min, transferring it to a desiccator, and then cooling, and weighing it (W). 2g of the pulverized sample was weighed into a dish (W1) and heated gently on the Bunsen burner and the charred mass was in a suitable condition and transferred to a muffle furnace at 550 º C and ashed for 6 hours (AOAC, 2000). The heating continued until all the carbon had been burnt away and a whitish substance was left. The dish plus ash was transferred to a desiccator, 'cooled, and weighed (W2) until a constant weight was obtained. The ash content of the sample was calculated as follows;

Average weight of Ash: Sample A = $\frac{\text{wt 1 + wt 2}}{2}$

$$\% \text{ Ash Content} = \frac{\text{Sample A}}{2} \times \frac{100}{1}$$

Determination of Crude Fibre

The crude fibre content of the samples was determined by sequential acid and alkali hydrolysis followed by ignition. Two grams (2 g) of a grounded sample (oven-dried) was collected in a 1-L beaker covered with a round-bottom flask containing cold water. The system maintains constant volume during boiling. The content of the beaker was refluxed to the boiling point with 250 ml of 1.25 % H_2SO_4. The beaker was then sealed with cotton, and the mixture was boiled on a heater for 30 min. Then, the content was filtered quickly in a Buchner funnel through Whatman No. 1 filter paper and washed to remove the acid. The acid-free residue was refluxed with 200 ml of 1.25 % NaOH solution at boiling point for exactly 30 min, maintaining the constant volume as before. Then, the mixture was filtered and washed with water to make the residue alkali-free. The residue was transferred to a crucible and dried in an oven at 105 ^{0}C until a constant weight was attained. The sample was then cooled in a desiccator and weighed (the residue contained ash and crude fibre). The sample was then ignited in a muffle furnace at 550^{0}C for 4 hours, after which it was cooled again in a desiccator. The weight of the ignited sample was recorded after subsequent cooling. The weight loss of the samples after ignition indicated the amount of crude fibre.

Calculation: The per cent of crude fibre is calculated using the following formula;

Weight of crude fiber = (Weight of crucible + crude fiber + ash) - (Weight of crucible + ash)

Determination of Crude Fat

2g of the pulverized samples was weighed into the thimble with porosity which permitted rapid passage of the solvent. The thimble was covered with deflated cotton wool, 50mL of petroleum ether was poured into the flask, and the Soxhlet extractor was fixed. The whole setup was put on electromantle and it was heated at 50 ^{0}C. The sample was extracted for 4 hours. The solvent was evaporated till no odor of solvent remained. Duplicate determination was done.

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Protein determination

The dried ground sample 2 g was taken into a Kjeldahl flask, and 18 ml of H_2SO_4 was added, after which 1g of $CuSO_4$ and 20 ml of conc. H_2SO_4. It was digested in the Kjeldahl digestion unit for 6 hours. The mixture was cooled down to room temperature. It was transferred into 50ml of 4% boric acid solution in a receiving flask and added to it 3-5 drops of mixed indicator and placed under the condenser of Kjeldahl. Distillation unit making sure that the condenser tube extends beneath the surface of the acid, in the flask, now added to the Kjeldahl flask was 50 ml water and 60 ml of 32% NaOH solution. It was distilled so that a volume of 200 ml was collected in the receiving flask. 0.1 N HCl was poured into the burette and titration was done. The reading was noted and the percentage of protein in it was determined as follows;

% Protein = % Nitrogen x 6.25.

Determination of carbohydrate

This was obtained by a different method in which the sum of moisture, fat, crude fibre, protein, and ash was subtracted from 100. This is referred to as estimated by the " different method"

Therefore, Carbohydrate : 100 – (Ash + Moisture + Fibre + Protein + Fat)

Digestion Methods for Plant Sample

Samples were digested as described by (Awofolu, 2005). 0.5g of powdered samples were weighed into a 100 cm^3 beaker. 5cm^3 concentrated nitric acid (HNO_3) and 2 cm^3 of perchloric acid ($HClO_4$), together with few boiling chips were added. The mixtures were heated at 70 ^{0}C for 15 minutes until a light-coloured solution was obtained. The sample solution was not allowed to dry during digestion. The contents were filtered into the 25 ml flask. The filtrate was allowed to cool to room temperature before dilution was made to the mark and the contents were mixed thoroughly by shaking. The digest was run on the PG 990 atomic absorption spectrometry machine.

High Performance Liquid Chromatography (HPLC)

Phytochemical analysis of the ethylacetate/methanolic extract of *C. haematocephala* was done by high-performance liquid chromatography (HPLC)

Instrument: Agilent 1100 HPLC with Online degasser, quarternary pump, auto liquid sampler, thermostated column compartment and a variable wavelength UV detector. Data for identification and quantification was processed on the CPU running on Agilent Chemstation software. The stationary Phase is Phenomenex Luna C18 (250 x 4.6 mm, 5 um) and the Column Temperature is 40^{o}C at the Flow Rate of 1.0 mL/min and Injection volume of 10 uL.

Mobile Phase A: 0.1%v/v Trifluoroacetic Acid in Water

Mobile Phase B: Acetonitrile

Standard Preparation:

25mg of Quercetin standard was dissolved in 25 mL of methanol while 25mg of Caffeine, Resorcinol and Gallic Acid were each dissolved in 25 mL of Mobile Phase A. 25mg of Theobromine standard was dissolved into a 25 mL flask. 5 mL of 0.1 N NaOH was added to dissolve completely then made up to the mark with water. Theobromine was calibrated separately. Gallic acid, Caffeine, Resorcinol and Quercetin were calibrated together by preparing a mixed standard stock.

Sample Preparation:

0.1 g of each sample was resuspended in 10mL of water/methanol (1:1), vortexed for 1min and filtered with a syringe filter (0.45um) into HPLC vials prior to HPLC analysis**.**

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Gas Chromatography and Mass Spectroscopy (GC-MS) analysis

The extracts were analysed using Shimadzu GC-MS-QP2010 Plus (Japan). The separations were carried out using a Restek Rtx-5MS fused silica capillary column (5%-diphenyl-95%-dimethylpolysiloxane) of 30×0.25 mm internal diameter (di) and 0.25 mm in film thickness. The conditions for analysis were set as follows; column oven temperature was programmed from 60–280 °C (temperature at 60°C was held for 1.0 min, raised to 180 °C for 3 min and then finally to 280°C held for 2 min); injection mode, Split ratio 41.6; injection temperature, 250°C; flow control mode, linear velocity (36.2 cm/sec); purge flow 3.0 ml/min; pressure, 56.2 kPa; helium was the carrier gas with total flow rate 45.0 ml/min; column flow rate, 0.99 ml/min; ion source temperature, 200°C; interface temperature, 250°C; solvent cut time, 3.0 min; start time 3.5 min; end time, 24.0 min; start m/z, 50 and end m/z, 700. The detector was operated in EI ionization mode of 70 eV. Components were identified by matching their mass spectra with those of the spectrometer database using the NIST computer data bank, as well as by comparison of the fragmentation pattern with those reported in the literature (Ololade and Olawore, 2018).

Antimicrobial Screening

Determination of Antibiotics Susceptibility Pattern of Bacteria Isolates

The susceptibility of isolated bacteria to conventional antibiotics was determined by Kirby-Bauer methods as described by CLSI (2020). Isolates were cultured at 37ºC for 24 hours and standardized to 0.5 MacFarland standards. One millilitre (1 ml) of the standardized inoculum was aseptically introduced into the surface of Mueller-Hinton agar plates and allowed to adapt to the plates for 30 minutes. Commercially available antibiotics discs inclusive of perfloxacin (30µg), Tarivid (10 µg), streptomycin (30 µg), Tarivid (10 µg), streptomycin (30 µg), septrin (30 µg), Chloramphenicol (30 µg), sparfloxacin (10 µg), ciprofloxacin (10µg), gentamycin (30 µg), augmentin (10 µg) and amoxicillin (30 µg) were used to determine the sensitivity of gram-negative bacteria to antibiotics (Yuan et al., 2017). The antibiotic discs were aseptically placed on the inoculated agar plates using sterile forceps. The aseptically covered plates were incubated at 37ºC for 18-24 hours. After the incubation, the diameter of the zones of inhibition was measured to the nearest millimetre (mm) using a transparent ruler, recorded and interpreted according to CLSI (2020) standard.

Determination of antimicrobial activity

The diffusion method was employed for the antimicrobial assay. The organisms used were obtained from the University of Ibadan, Microbiology Department and Microbiology Laboratory of Bowen University, Iwo, Osun State. The Gram-negative bacteria used were; *Escherichia coli* (PQ049988), *Campylobacter jejuni* (RM1875), *Vibrio cholera* (ATCC14028), *Klebsiella pnuemoniae*, *Pseudomonas aeruginosa.* The Gram-positive bacteria used were; *Staphylococcus aureus* (ATCC25923), *Clostridium difficile*. The Fungal specie used was; *Candida Albicans* (ATCC2147) & *Candida tropicalis*. The experiment culture was generated by immersing a loop full of bacteria cells in peptone water and incubating for 24 hours at 37 degrees Celsius (Mulaw *et al.*, 2019). Following that, the optical density was measured at 625nm and standardised using the 0.5 McFarland standard (Souza *et al*., 2020). The Kirby-Bauer disc diffusion method was employed to conduct an antimicrobial susceptibility test, with Muller-Hinton agar (LABM) as the medium. Concentrations of C. haematocephala extract (250 and 100 mg/ml) in normal saline are employed in triplicates. The antibacterial and antifungal potential of the plant extracts were calculated using the mean of the diameter of the zone of growth inhibition against the test organisms. Ciprofloxacin, Augmentin, and Gentamycin were used as controls for bacteria, fluconazole and ketoconazole were used as controls for fungi.

Membrane Perturbation assay for *Calliandra haematocephala*

Preparation of Human Erythrocytes

Fresh human blood was prepared according to the method described by Oyedapo *et al.* (2010) as reported by Godwin *et al*. (2023). The collected blood was poured gently into clean centrifuge tubes and centrifuged at 3000 rpm for 10 minutes. The supernatant containing the leukocytes and plasma was carefully removed with

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dry clean Pasteur pipettes leaving behind packed erythrocytes in the centrifuge tubes (Duchnowicz *et al*., 2021). The packed erythrocyte was re-suspended in fresh isosaline (0.85% w/v NaCl), mixed carefully, and centrifuged at 3000 rpm for 10 minutes. The supernatant was carefully removed and the process was repeated until a clear supernatant was obtained. To 2 ml of the packed erythrocytes, isosaline (98 ml) was added to make 2% (v/v) red blood cells.

Membrane Perturbation Assay

The membrane perturbation or stabilizing assay was carried out using the procedure of Oyedapo *et al*. (2010) as reported by Godwin *et al*. (2023). The assay mixture consisted of hyposaline (0.42% w/v NaCl; 0.5ml), sodium phosphate buffer (0.15M, pH 7.4, 1.0ml) varying volumes of isosaline (0.85% w/v NaCl) and 2% (v/v) human erythrocytes (0.5ml). The drug control was prepared to contain all the reagents except 2% (v/v) human erythrocytes. The blood control, on the other hand, contained all the reagents except the extract or reference drug. The assay mixture was mixed gently and incubated at 56^{o}C for 30 mins. Test tubes were allowed to cool and thereafter centrifuged at 3000 rpm for 5 mins, and the supernatant was collected and read at 560 nm against the blank. The percentage membrane stabilizing activity was estimated from the expression:

Control represents 100% lysis or perturbation of the human red blood cells.

$$100 - \frac{\{(\text{drug test value} - \text{drug control value})\} * 100}{\text{Control}}$$

Animal study

Prior to the start of the experiment, an ethical clearance for performing the experiment on animals was obtained from the university animal research ethics committee (UAREC) and the reference number; UNIMED-AREC/Apv/2024/57 was generated as the approved I. D No. for the study. Thirty mature male Wistar rats weighing 150-200g were provided by the animal house. The rats were individually housed in cages and maintained under controlled environmental conditions such as temperature (20±2 %), relative humidity (45-55 %) and 12 h dark/light cycle. All rats were fed with rodent pellet diet and water *ad libitum* under strict hygienic conditions. After one week of acclimatisation according to the set criteria, the rats were randomly divided into seven different groups, each having six rats.

Induction of experimental urolithiasis in Wistar rats

Calcium oxalate urolithiasis was induced in the rat by free access to drinking water containing 1% (v/v) ethylene-glycol (EG) for 28 days.

In-vivo Study protocol

Group I: (Control) –Animals in this group were allowed access to distilled water *ad libitum* throughout the experiment for 28 days.

Group II: (Induced) –Animals in this group were allowed access to drinking water containing 1% EG ad libitum for 28 days.

Group III: (Treated) –Animals in this group were allowed access to drinking water containing 1% EG and administered standard Tamsulosin hydrochloride (5 μg/kg) orally for 28 days.

Group IV: (Treated) – Animals in this group were allowed access to drinking water containing 1% EG and *Calliandra haematocephala* extract (50 mg/kg) orally for 28 days.

Group V: (Treated) – Animals in this group were allowed access to drinking water containing 1% EG and *Calliandra haematocephala* extract (100 mg/kg) orally for 28 days.

The groups I-V received 1% ethylene-glycol in drinking water *ad libitum* for 28 days, respectively, to generate CaOx deposition into kidneys and induce urolithiasis. All animals were placed in different metabolic cages on the twenty-eighth day of calculi induction and treatment. Blood was collected via cardiac puncture after

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euthanizing with cervical dislocation; serum was separated by centrifugation at 10,000 g for 10 min. The animals were sacrificed by cervical dislocation and kidney tissues were removed and stored at -20 ◦C till the analysis period (Olayeriju *et al*., 2021).

Biochemical Assay

Urine and plasma nephrochemical assays of creatinine, urea, total protein, albumin and calcium were carried out as previously described by Olayeriju *et al*., (2020).

At the end of the study, rats were fasted overnight, and anaesthetised with thiopental sodium at 50 mg/kg (Sajovic *et al.*, 2022). Urine samples were collected and blood samples were also collected by cardiac puncture, centrifuged at 200 x g for 15 min, after 30 min of collection and stored at -20 °C until analysis. The kidney was collected and stored at -20 °C until use. Serum was obtained by centrifugation of blood at 3000rpm for 15 min. The serum obtained was used to examine the following biochemical tests: Urea, Creatinine, and total protein using commercial kits (Biosystems S.A Costa Brava 30, Barcelona, Spain). Parameter concentrations were determined using an ultraviolet (UV) visible spectrophotometer at 571 nm wavelength (Milton Roy spectronic 601 Spectrophotometer, USA).

Histological Analysis

The kidneys were fixed in 10% phosphate-buffered formalin for 24 hours at room temperature and then embedded in paraffin. (Sikarwar et al., 2019) Sections were prepared with 3 µm thickness and stained with Masson's trichrome stain. All the slides were scanned by Nanozoomer digital pathology and were analyzed by Metamorph offline version 7.7.0.0 software. Glomeruli (90 to 120 per slide) were evaluated (scored from 0 to 4) on the basis of the degree of glomerulosclerosis as previously described. The slides were viewed under the light microscope to study the light microscopic architecture of the kidney.

Statistical Analysis

All data were expressed as Mean ± Standard Error of the Mean (SEM)**.** Statistical comparisons between groups were performed using one-way Analysis of Variance (ANOVA) followed by Tukey's post hoc test to determine significant differences. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant. Statistical analysis was conducted using GraphPad Prism 10. Groups that do not share the same letter were considered significantly different based on Tukey's test results.

RESULTS

Table 1 Qualitative Preliminary Phytochemical Screening of the leaf extract

S.No.	Phytoconstituents	*C. hematocephala*
1.	Alkaloids	+
2.	Phenol	+
3.	Tannins	+
4.	Carbohydrate	+
5.	Protein	+
6.	Steroids	+
7.	Sterols	+
8.	Glycosides	+
9.	Flavonoids	+
10.	Terpenoids	+
11.	Saponins	+

KEY: + = Present - = Absent

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The phytochemical analysis of *C. haematocephala* showed the presence of various phytochemical compounds in the extract. Phytochemicals such as alkaloids, glycosides, steroids, flavonoids, saponins, tannins, and terpenoids are present in the methanol/ethyl acetate extract which may help in treating many diseases.

Table 2 Quantitative phytochemical analysis of *C. haematocephala*

Plant sample	Quercetin (mgQE/gram)	Gallic acid (mg/g in GAE)	Alkaloids (µgAtropine/g)	Total antioxidant Capacity (TAC) (mmol Trolox/kg)
C. haematocephala	112.17 ± 1.95	129.67 ± 1.32	150.65 ± 1.96	14.51 ± 0.15

Values are reported as Mean ± Standard error of the mean (SEM)

The proximate analysis of the extract of *Calliandra haematocephala* shown in Table 3 indicated the percentage of moisture as 3.50 % and ash content of 6.70 %, while crude protein is 13.13 %, crude fibre is 4.50%, crude fat is 1.75 %, and carbohydrate is 70.42 %.

Table 3 Proximate analysis of *C. haematocephala*

Proximate composition	*C. haematocephala* (%)
Moisture	3.50
Crude Protein	13.13
Crude Fat	1.75
Ash	6.70
Crude Fibre	4.50
Carbohydrate	70.42

The most common macroelement in *C. haematocephala* are zinc (1.355ppm), manganese (1.308ppm), copper(1.200ppm), magnesium(1.257ppm), selenium(1.020ppm), calcium(1.088ppm) and chromium(1.019ppm) while arsenic (0.061ppm), mercury(0.096ppm), lead(0.0833ppm), iron(0.093ppm), potassium (0.655ppm) and sodium(0.398ppm) were available in little quantity in the plant extract.

Table 4 Mineral Composition of *Calliandra haematocephala*

Mineral composition	*Calliandra haematocephala* (ppm)mean±SD
Arsenic (As)	0.061±0
Mercury (Hg)	0.091±0.003
Selenium (Se)	1.020±0.00
Manganese (Mn)	1.308±0.002
Zinc (Zn)	1.355±0.00
Copper (Cu)	1.200±0.001
Lead (Pb)	0.089±0.008
Chromium (Cr)	1.019±0.001
Iron (Fe)	0.079±0.009
Calcium (Ca)	1.088±0.00
Magnesium (Mg)	1.255±0.002
Potassium (K)	0.655±0.001
Sodium(Na)	0.398±0.0006

Values are reported as mean ± Standard Deviation (SD)

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The table showed five predominant constituents in *Calliandra haematocephala*. In *Calliandra haematocephala*, a peak with a retention time of 5.197 min was identified to be gallic acid. The retention time identified for theobromine, resorcinol, caffeine and quercetin were 7.183, 7.625, 9.615 and 15.862 respectively (Table 5).

Table 5 The HPLC analysis of the compounds present in *Calliandra haematocephala*

Peak	Compound	Wavelength (nm)	Ret Time (min) *Calliandra haematocephala*
1	Gallic acid	271	5.197
2	Theobromine	271	7.183
3	Resorcinol	271	7.625
4	Caffeine	271	9.616
5	Quercetin	271	15.862

9 compounds were identified in the leaf extract of *Calliandra haematocephala* with a total percentage composition of 100.01 (Table 6). The compounds include Squalene (0.162%), α-d-Mannofuranoside, methyl (0.312%), 14,17-Octadecadienoic acid, methyl ester (0.019%)**,** Phytol (0.019%) and so on.

Table 6 GC-MS result of Ethylacetate/Methanolic Leaf Extract of *C. haematocephala*

Compounds	**Percentage Composition (%)**	**Retention Index**
Ethanol, 2-[2-(2-butoxyethoxy)ethoxy]-	0.069	1486
d-Gala-l-ido-octonic amide	0.019	2442
Morpholine, 4-methyl-, 4-oxide	0.057	0
d-Glucitol, 4-O-nonyl-	0.020	2496
Squalene	0.162	2914
.alpha.-d-Mannofuranoside, methyl	0.312	1667
Hexadecanoic acid, methyl ester	0.031	1878
14,17-Octadecadienoic acid, methyl ester	0.019	2075
Phytol	0.019	2045
Percentage Total	**100.01**	

Antimicrobial Screening

Antimicrobial activity was tested in *C. haematocephala* ethyl acetate/methanol extract against gram-positive bacteria; *Staphylococcus aureus* and *Clostridium difficile* and against gram-negative bacteria; *Escherichia coli*, *Campylobacter jejuni* and *Vibrio cholera*. And also against fungal agent, *Candida albicans*. It was observed that *C. haematocephala* gave low zone of inhibition against all the bacteria strain selected for the study, including *Vibrio cholera* (6 mm), *Clostridium difficile* (7 mm), *Escherichia coli* (10 mm), *Campylobacter jejuni* (7 mm) and *Staphylococcus aureus* (5 mm) at 250 mg/mL. At a much lower inhibition at 100 mg/mL for *Vibrio cholera* (5 mm), *Campylobacter jejuni* (4 mm) and *Staphylococcus aureus* (2 mm).

Antifungal Activity

From Table 9, it was observed that *C. haematocephala* showed low inhibition of 6 mm at 250 mg/mL and 5 mm at 100 mg/mL.

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Table 7 Antimicrobial sensitivity Test

	Bacteria Isolates Zones of Inhibition (mm)				
Antibiotics	***Vibrio cholerae***	***Campylobacter Jejuni***	***Escherichia coli***	***Staphylococcus aureus***	***Clostridium difficile***
SXT (30µg)	21.33±0.34	11±0.58	18.33±0.33	19.67±0.33	19.67±0.33
CH(30µg)	14.67±0.33	10±0.58	12.67±0.33	9.667±0.33	9.333±0.33
SP(10µg)	10.67±0.333	0.667±0.33	20.33±0.33	8.667±0.33	0
CPX(30µg)	19.33±0.333	15.33±0.33	14.67±0.33	0	0
AM(30µg)	8.333±0.333	4.667±0.33	18.33±0.33	11.33±0.33	4.333±0.33 ±0.33
AU(10µg)	10.33±0.333	0±0	7.667±0.33	11.67±0.33	8.333±0.33
CN(30µg)	12.67±0.333	12.67±0.33	10.67±0.33	18.67±0.33	20.33±0.33
PEF(30µg)	22.67±0.333	16±0.58	19.33±0.33	10.67±0.33	1.667±0.33
OFX(10µg)	23±0.577	17.33±0.33	20.67±0.33	11.67±0.33	10.33±0.33
S(30µg)	17.67±0.333	1.667±0.33	18.67±0.33	18.33±0.33	2.667±0.33
		Values recorded as Mean ± SEM			

Key: CN: Gentamycin (30 ug), OFX: Tarivid (10 ug), SXT: Septrin (30 ug), SP: Sparfloxacin(10 ug), AM: Amoxacillin (30 ug), CH: Chloramphenicol (30 ug), AU: Augmentin (10 ug), S: Streptomycin (30 ug), PEF: Perfloxacin (10 ug), CPX: Ciprofloxacin (30 ug)

Table 8 Antibiotic activity of *C. haematocephala* extract

ORGANISM	CONCENTRATIONS(mg/ml) of Ciprofloxacin		N-Saline	CONCENTRATIONS(mg/ml) of *C. haematocephala* Extract	
	250 mg/ml	100 mg/ml		250 mg/ml	100 mg/ml
Vibrio cholera	17 mm	10 mm	0	6 mm	5 mm
Clostridium difficile	20 mm	18 mm	0	7 mm	3 mm
Escherichia coli	20 mm	16 mm	0	10 mm	5 mm
Campylobacter jejuni	20 mm	16 mm	0	7 mm	4 mm
Staphylococcus aureus	17 mm	16 mm	0	5 mm	2 mm

Key note: - = No inhibition, 6 – 9mm = low inhibition, 10 – 15 mm = Moderate inhibition, and ≥15 mm = high inhibition

Table 9 Antifungal activity of the plant extract

Antifungal agents	*Candida albicans* activity on *C. haematocephala* Extract	
	250mg/ml	100mg/ml
Ketoconazole	8 mm	6 mm
Fluconazole	6 mm	4 mm
Extract	6 mm	5 mm
Normal Saline	0	0

Key note: - = No inhibition, 6 – 9mm = low inhibition, 10 – 15 mm = Moderate inhibition, and ≥15 mm = high inhibition

Membrane Perturbation

The results indicate that *C. haematocephala* demonstrates a variable but generally increasing stability percentage with increasing concentrations, reaching maximum stability at the highest concentration. In contrast, Tamsulosin HCl exhibited a steady increase in stability at the same concentration. At the lower concentrations, the extracts and the standard drug showed similar stability percentages, with Tamsulosin HCl. However, as the concentration increased, *C. haematocephala* began to exhibit superior stability but Tamsulosin HCl increased at a slower rate.

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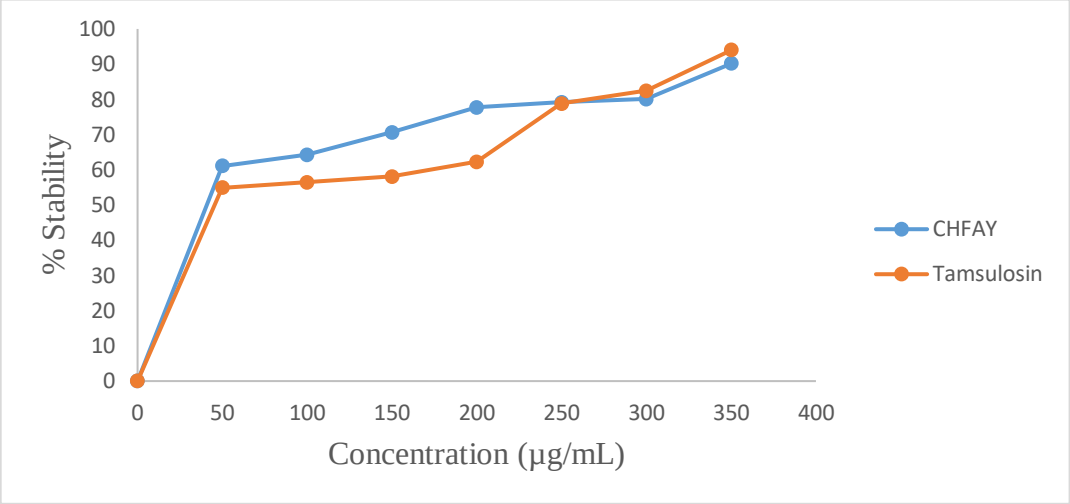

Fig. 2: Membrane Stabilizing Profiles of C. haematocephala and Tamsulosin (Standard Drug) Extracts on Stressed and Perturbed Human Erythrocytes. Each value represents the Mean ± SEM of 3 readings.

Table 10 presents the biochemical parameters in ethylene glycol-induced urolithiasis rats. Creatinine levels significantly increased across all groups compared to the control, with the highest elevation in the induced group. Urea levels were significantly ($p< 0.05$) reduced in the induced group but increased in the treated groups. Uric acid levels were significantly elevated in the induced group, while treatment with the standard drug and plant extract reduced these levels. Serum electrolytes (calcium, chloride, potassium, and sodium) showed significant variations. Calcium and chloride levels decreased in the induced group but improved in the treated groups. Potassium increased in the induced group but was reduced by both the standard drug and plant extract, with a dose-dependent effect. Sodium levels declined in the induced group but increased upon treatment. Total protein and albumin levels significantly declined in the induced group but improved with treatment, showing a dose-dependent response. Glucose levels were elevated in group B but significantly reduced in the standard drug and extract-treated groups. Myeloperoxidase (MPO) activity increased in the induced group but was significantly reduced with treatment. SOD and CAT levels declined in the induced group but improved with the standard drug and extracts, whereas malondialdehyde (MDA) levels were significantly elevated in the induced group but reduced with treatment. All results were statistically significant ($p<0.05$).

Table 10 The Biochemical Parameters of Ethylene-glycol induced Urolithiasis Rat.

Parameter	Control	Induced group	Standard drug	*C. haematocephala* 50mg/kg	*C. haematocephala* 100mg/kg
Creatinine	0.46 ± 0.02^{a}	0.78 ± 0.02^{b}	0.60 ± 0.01^{c}	0.66 ± 0.01^{f}	0.53 ± 0.02^{g}
Urea	29.66 ± 0.84^{a}	18.62 ± 1.11^{b}	22.11 ± 0.63^{a}	25.51 ± 0.41^{a}	26.26 ± 0.77^{a}
Uric acid	1.15 ± 0.01^{a}	3.70 ± 0.12^{f}	2.99 ± 0.03^{g}	3.14 ± 0.04^{d}	2.34 ± 0.10^{e}
Calcium	6.12 ± 0.27^{a}	2.98 ± 0.18^{d}	6.43 ± 0.15^{a}	4.56 ± 0.17^{b}	5.09 ± 0.09^{b}
Chloride	92.25 ± 2.74^{e}	50.70 ± 1.20^{d}	57.87 ± 1.20^{d}	63.31 ± 1.94^{b}	71.45 ± 0.96^{a}
Potassium	8.48 ± 0.57^{a}	20.16 ± 0.86^{c}	11.97 ± 0.71^{a}	10.89 ± 0.27^{a}	5.41 ± 2.73^{a}
Sodium	129.75 ± 1.49^{c}	91.09 ± 2.89^{d}	112.69 ± 0.63^{a}	113.92 ± 0.61^{a}	120.13 ± 0.67^{a}
Total protein	45.91 ± 0.69^{a}	34.93 ± 1.54^{b}	38.50 ± 0.74^{b}	41.53 ± 0.45^{c}	46.40 ± 1.16^{a}
Albumin	32.94 ± 0.72^{a}	24.44 ± 0.53^{e}	28.68 ± 0.49^{b}	36.11 ± 0.24^{c}	38.29 ± 0.72^{c}
Glucose	171.92 ± 0.56^{a}	187.33 ± 1.04^{d}	173.61 ± 0.88^{a}	177.93 ± 0.61^{c}	167.71 ± 0.99^{a}
MPO activity	33.28 ± 1.29^{d}	68.66 ± 1.88^{c}	59.17 ± 1.32^{a}	45.96 ± 0.94^{a}	42.25 ± 0.87^{a}
SOD	0.31 ± 0.20^{a}	0.11 ± 0.00^{c}	0.14 ± 0.01^{c}	0.23 ± 0.02^{a}	0.25 ± 0.02^{a}
CAT	0.47 ± 0.01^{b}	0.23 ± 0.01^{c}	0.35 ± 0.01^{b}	0.27 ± 0.01^{c}	0.43 ± 0.01^{b}
MDA	5.01 ± 0.31^{d}	11.89 ± 0.60^{c}	9.34 ± 0.17^{b}	8.87±0.17b	7.00 ± 0.36^{a}

Values presented in Mean±SEM. Different letters indicate that data are statistically significant while similar letters indicate that data are not statistically significant @ $p< 0.05$.

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Figure 1 shows the effect of Ethylacetate/methanolic extract of *C. haematocephala* on general Histopathological examination of the cross-section of the kidney

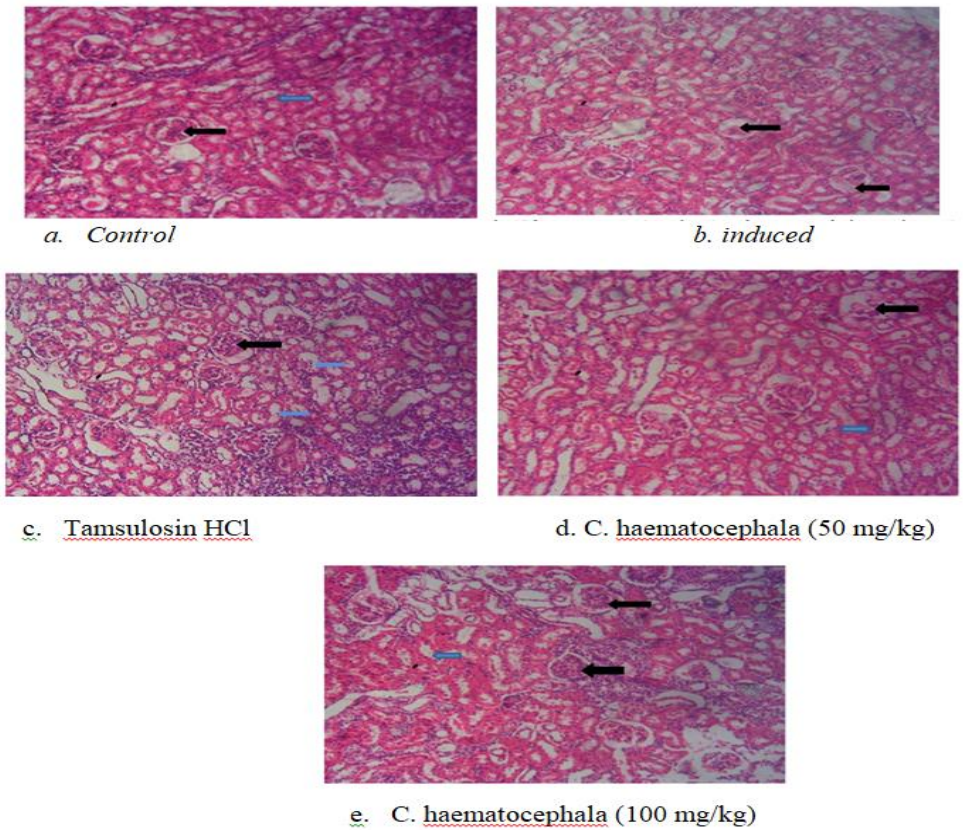

Figure 1. Representation of light micrographs of the cross-section of the kidney tissues.

a: The control group showed normal kidney histoarchitecture b: The induced group showed a reduced glomerulus. c: Tamsulosin treated group showed normal kidney histoarchitecture. d: C. haematocephala treated group (50 mg/kg) showed normal histoarchitecture and degenerated kidney tubule. e. C. haematocephala treated group (100 mg/kg) showed normal kidney histoarchitecture and kidney tubule with no inflammation.

DISCUSSION

Qualitative Preliminary Phytochemical Analysis

It is proven that both flavonoids and tannins possess greater antimicrobial activities (Sharma *et al*., 2016: Silva *et al*., 2018). Flavonoids are hydroxylated phenolic compounds known to be synthesized by plants in response to microbial infection. Tannins have great antibacterial activity by binding with proline-rich proteins that interfere with protein synthesis (Luciana *et al.*, 2023). Alkaloids are secondary metabolites that are effective as anti-depressants (Tian *et al.*, 2023). *C. haematocephala* extract shows a positive result for the Ferric chloride test which indicates the presence of tannin and it is similar to the work of Vadera *et al*., 2022. The presence of alkaloids, tannins, and flavonoids in *C. haematocephala* helps it to inhibit ion channels, cleave the biomembrane, and damage microtubules and microfilament. In the context of kidney stone elimination, these chemical compounds especially saponins, flavonoids, alkaloids, phenolic acids, and terpenoids help in the relaxation of smooth muscles in the ureter and this leads to ease in passing out stones from the kidney. They also have the ability to reduce inflammation and muscle spasms.

Quantitative phytochemical analysis

Quercetin is a natural flavonoid antioxidant, specifically, it is a flavonol and was found to be present in a quantifiable amount in *C. haematocephala* (112.17 mgQE/g). Quercetin may help protect against heart disease,

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inflammatory disorders, and cancer (Rahul *et al.,* 2018). Statilko *et al.,* (2024) confirmed the presence of quercetin when they evaluated the phytochemical components of four varieties of Cabbage. Gallic acid is a trihydroxy benzoic acid and is classified as phenolic acid. It is a high antioxidant due to its three hydroxyl groups. Gallic acid is abundant in *C. haematocephala* (129.67 mg/g). It has been reported that 18 brassica species and some medicinal plants contain alkaloids as the typical secondary cruciferous metabolites (Heinrich *et al.,* 2021). This is evident from this study with *C. haematocephala* (150.65 µg/g). The total antioxidant capacity (TAC) using FRAP reagent was also reported to be 14.51 mmol/kg for *C. haematocephala*.

Proximate and Mineral Composition

Notably, the proximate analysis of the extract of *Calliandra haematocephala* shown in Table 3 indicated the percentage of moisture as 3.50 % and ash content of 6.70 %, while crude protein is 13.13 %, crude fibre is 4.50%, crude fat is 1.75 %, and carbohydrate is 70.42 %. This analysis suggests that the presence of these secondary metabolites has the potential to exert biological effects. In the soil and air, many contaminants are present, which are absent in plants during growth and development and can pose a global hazard to human health. The results of the analysis of the elements in the tested extract of *C. haematocephala* using an atomic absorption spectrophotometer are shown in Table 4. This study confirmed that the extracts are rich in macro and microelements (Mizrob *et al*., 2023). The significantly higher concentration of zinc in relation to copper in *C. haematocephala* is due to the antagonistic interaction of the adoption of the two elements (Zn and Cu) which is evidenced in the fact that the presence of one reduces the presence of the other element, this is confirmed in other research (Mizrob *et al*., 2023). The relatively high concentration of magnesium and calcium in *C. haematocephala* is characteristic of all investigated extracts.

HPLC Analysis

In *Calliandra haematocephala*, a peak with a retention time of 5.197 min was identified to be gallic acid. The retention time identified for theobromine, resorcinol, caffeine and quercetin were 7.183, 7.625, 9.615 and 15.862 respectively. These compounds are notable for various therapeutic effects like anti-inflammatory and antioxidant properties.

GC-MS Analysis

This analysis is pivotal in identifying potential phytochemicals that may contribute to the medicinal properties of the plants, especially in the context of urolithiasis. 9 compounds were identified in the leaf extract of *Calliandra haematocephala* with a total percentage composition of 100.01. The compounds includes, Squalene (0.162 %) a triterpene is widely recognised for its antioxidant and anti-inflammatory properties (Matsuda *et al.,* 2017). α-d-Mannofuranoside, methyl (0.312%) is a sugar derivative that may exhibit prebiotic properties, promoting gut health and potentially influencing systemic inflammation (Zhang *et al.,* 2021). 14,17-Octadecadienoic acid, methyl ester (0.019%) is a polyunsaturated fatty acid that has been studied for its anti-inflammatory effects, which may be relevant in the management of urolithiasis (Jiménez-Nevárez *et al*., 2023). Phytol (0.019%) is known for its antioxidant properties and has been implicated in various therapeutic effects, including anti-inflammatory actions (Santos *et al*., 2013). Other compounds identified have various other benefits but their role in urolithiasis is not clear enough.

Antibacterial and Antifungal Activity.

Antimicrobial activity was tested in *C. haematocephala* ethyl acetate/methanol extract against gram-positive bacteria; *Staphylococcus aureus* and *Clostridium difficile* and against gram-negative bacteria; *Escherichia coli*, *Campylobacter jejuni* and *Vibrio cholera*. And also against fungal agent, *Candida albicans*. In our study, the ethanolic extract of leaves of *Calliandra haematocephala* showed antibacterial activity against both gram positive and gram negative strains and against the fungal strains in all doses. It was observed that *C. haematocephala* gave low zone of inhibition against all the bacteria strain selected for the study, including *Vibrio cholera* (6 mm), *Clostridium difficile* (7 mm), *Campylobacter jejuni* (7 mm) and *Staphylococcus aureus* (5 mm) and *Candida albicans* (6 mm) at 250 mg/mL. A much lower inhibition at 100 mg/mL for *Vibrio cholera* (5 mm), *Campylobacter jejuni* (4 mm), *Staphylococcus aureus* (2 mm) and *Candida albicans* (5 mm)

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was observed. However, it is more particularly effective against *Escherichia coli* (10 mm) in our study. This result pattern aligns with the work of Irudhaya *et al*., 2017, wherein some selected bacteria strain was tested against the ethanolic extract of *C. haematocephala*. The presence of flavonoids and tannins may be responsible for the antibacterial activity.

Membrane Perturbation

The stability of cellular membranes is crucial for maintaining cellular integrity and function. In this study, the membrane perturbation effects of *C. haematocephala* leaf extract were evaluated and compared with Tamsulosin HCl, a standard drug commonly used in the management of urolithiasis. The results indicate that *C. haematocephala* demonstrate a variable but generally increasing stability percentage with increasing concentrations, reaching maximum stability at the highest concentration. In contrast, Tamsulosin HCl exhibited a steady increase in stability at the same concentration. This therefore highlights the potential of *C. haematocephala* as an effective compound in maintaining the integrity of the membrane (Anosike *et al*., 2012). This property is essential in urolithiasis to ensure the preservation of cellular function for renal health (Ghosh *et al*., 2020: Alam *et al*., 2021). The mechanism by which the membrane gained stability in this study could be due to the incorporation of bioactive compounds in *C. haematocephala* into the membrane structure to strengthen the integrity of the membrane (Mansour *et al.,* 2018).

This could also be attributed to the phytochemical composition of *C. haematocephala* which contains various antioxidants and compounds that may contribute to membrane protection. These findings suggest a promising role for *C. haematocephala* in therapeutic applications, particularly in enhancing cellular resilience against perturbation.

Biochemical Assay

The study demonstrated that *Calliandra haematocephala* extract effectively mitigated kidney dysfunction and oxidative stress induced by ethylene glycol. The induction led to increased levels of creatinine, uric acid, potassium, glucose, MPO activity, and MDA while decreasing calcium, urea, sodium, chloride, total protein, albumin, SOD, and CAT, indicating severe renal impairment (Abo-elhamid *et al*., 2016). The plant extract, administered at 50 mg/kg and 100 mg/kg, reversed these biochemical alterations in a dose-dependent manner, improving renal function and reducing oxidative damage. *C. haematocephala* extract notably restored serum albumin levels, which were significantly reduced in the induced group, suggesting its role in reducing inflammation and maintaining metabolic balance (Soeters *et al*., 2019). The extract also improved calcium levels, preventing calcium oxalate stone formation, and exhibited a diuretic effect by increasing urine output. Additionally, the extract significantly reduced MDA levels, highlighting its antioxidant potential in combating lipid peroxidation (Butterweck et al., 2009). These findings confirm the nephroprotective and anti-urolithiasis effects of *C. haematocephala*, making it a promising natural alternative for managing kidney stone formation and oxidative kidney damage.

Histopathological Assay

The histological analysis of kidney tissues from *Calliandra haematocephala*-treated groups showed a dose-dependent protective effect against ethylene glycol-induced renal damage. At 100 mg/kg, *C. haematocephala* maintained normal kidney histoarchitecture, with intact glomeruli, normal renal tubules, and no signs of inflammation, similar to the control group (Wahid *et al.,* 2023). This indicates its potential in preserving kidney structure and function (Patel *et al*., 2012). However, at 50 mg/kg, some pathological changes, including shrunken glomeruli, enlarged tubules, and necrosis, were observed, suggesting that a higher dose offers better nephroprotection (Schelling, 2016). These findings highlight the ameliorative role of *C. haematocephala* in preventing kidney damage in urolithiasis.

CONCLUSION

This study validates the multifaceted therapeutic potential of *Calliandra haematocephala* integrating their phytochemical abundance with biological efficacy. The findings provide a robust foundation for the

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development of novel phytotherapeutics for managing urolithiasis and combating antimicrobial resistance. The 5 predominant constituents from the HPLC and 9 compounds from GC-MS present in *C. haematocephala* may contribute to the biological properties of the plants, especially in the context of urolithiasis. The plant also exhibited good membrane stabilizing potential thus protecting the membrane from oxidative damage. Through the utilisation of the special potency of this plant, this research opens the door to creative and long-lasting remedies for diseases like urolithiasis.

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