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ANTILITHIATIC ACTIVITY OF LEAF EXTRACT OF *CITRUS MEDICA* ON SODIUM OXALATE UROLITHIAIS–*IN-VITRO* AND *IN-VIVO* EVALUATION

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Keywords:

Citrus medica, Urolithiasis, Sodium oxalate, Nucleation, Aggregation assay

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ABSTRACT: Urolithiasis is a common urological disorder responsible for serious human affliction and cost to society with a high recurrence rate. The aim of the present study was to systematically evaluate the leaf extract of *Citrus medica* using suitable *in-vitro* and *in-vivo* models to provide scientific evidence for its antilithiatic activity. Cystone has been suggested to be beneficial in urolithiasis, as it corrects the crystalloid, colloid stability. It is evaluated that flavonoids, triterpenoids and saponins from different plants resulted in the antilithiatic activity. To explore the effect of *Citrus medica* on sodium oxalate crystallization, *in-vitro* assays like crystal nucleation and aggregation were performed. The biochemical parameters like calcium, oxalate, magnesium, phosphate, sodium, and potassium were evaluated in urine, serum, and kidney homogenates. Histopathological studies were also done to confirm the biochemical findings. *In-vitro* experiments with *Citrus medica* showed concentration-dependent inhibition of sodium oxalate nucleation and aggregation. In the *in-vivo* model, *Citrus medica* reduced both sodium and oxalate supersaturation in urine, serum, and deposition in the kidney. The biochemical results were supported by histopathological studies. The findings of the present study suggest that *Citrus medica* has the ability to prevent nucleation and aggregation growth of sodium oxalate crystals. *Citrus medica* has a better preventive effect on sodium oxalate stone formation, indicating its strong potential to develop as a therapeutic option to prevent recurrence of urolithiasis.

INTRODUCTION: Renal lithiasis is the stage where calculi are formed or located anywhere in the urinary system, or the process of formation of stones in the kidney, bladder, and ureters urinary tract [1]. Urinary calculi disease is increasingly dominant, with an eternity risk of about 12% in men and 6% in women. Age of onset of an initial stone episode for men rises from their 20's and excessive at age 40-60 years, with an occurrence of 3 cases/1000 population per 365 days [2].

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Renal calculi occurrence is a complicated method that outcomes from several physicochemical activities, including incredible saturation, nucleation, increase aggregation, and kept inside the renal tubules. Among the available treatments, ESWL, and drug treatment, which revolutionized urological practice, nearly turned out to be the same old procedure for casting off kidney stones [3]. Cystone has been suggested to be beneficial in urolithiasis, as it corrects the crystalloid and colloid stability and also acts through disintegrating calculi and crystals [4]. It is evaluated that flavonoids, triterpenoids, saponins from different plants resulted in antilithiatic activity [5].

Besides, the globe Health Organization has dependable and reliable that over seventy-five

percent of the world's population still depend upon an organism of kingdom Plantae-derived medicines, typically obtained from ancient healers for good health.

Citrus medica Linn. ordinarily considered as the Citron in English and bijapura in ayurvedic literature, is bush or little tree half dozen [6]. Completely different elements of citrus species were screened against infective organisms [7-11]. Numerous citrus species were far-famed for various activities like antimicrobial [12], antidiabetic, hypo-glycaemic [13], antiulcer [14], fungitoxocity [15], estrogenic [16]. Fruits of *Citrus medica* were used for antilithiatic activity [17-18] and antioxidant activity. The objective of the present work is to assess the leaf extract for *in-vitro* and *in-vivo* antilithiatic activity.

MATERIALS AND METHODS:

Plant Collection: The leaves of *Citrus medica* were collected from Bibipet Mandal, Kamareddy district. This material was known and well-tried to be real and validated by Botanist in Dec 2018 and was identified (CM 20122018) by academician and Head of Department of Botany of Government Degree College, Kukatpally.

Preparation of Methanolic Extract of *Citrus medica* Leaves: The small-grained leaf material was successively extracted in methanol by the soxhlation technique. It is continued again and again, thus to get efficient, economical extraction. The extract obtained was kept in desiccators to avoid excess moisture, finally moved to airtight containers for further future use.

Preliminary Phytochemical Investigation of Plant: The extract was subjected to preliminary phytochemical investigations to identify various phytoconstituents present in the leaves of *Citrus medica.*

Animals: An inbred colony of adult Wistar rats (150-200gm) is procured from Jeeva life sciences laboratory, and they are used for the present study. They were placed at relative humidity 45-55% in propylene cages at 25 ± 2°C under 12 hour light and dark cycles. All the animals were fed with standard feed *ad libitum*, water and were acclimatized to the laboratory conditions for a week. IAEC has approved all experimental pharmacological protocols (approval no: 1175/PO/Re/S/08/CPCSEA).

Acute Toxicity Testing: The animals were fasted overnight, providing only water, after which extract was administered to the respective groups orally at the dose level of 2000 mg/kg body weight. The groups were observed continuously for behavioral, neurological, autonomic profiles, and any lethality.

***In-vitro* Evaluation of the Antilithiatic Activity:**

Nucleation and Aggregation Assay: Percent decrease of the nucleation-aggregation assay was measured by the coincidental model delineated by Sharma *et al.* In-short, a freshly prepared sample of 10 mM calcium chloride dihydrate containing 200 mM sodium chloride and 1.0 mM sodium oxalate having 10 mM Na acetate trihydrate was set with pH 5.7, at 37 °C using a water bath. For crystallization experiments, the beaker was placed within the hot plate magnetic stirrer (Model 2MLH, REMI) with 25 mL of sodium oxalate solution, which was maintained at 37 °C and by 800 revolutions per minute perpetually stirred. An additional (25 mL) calcium chloride solution was added, and initially distilled water/standard (Cystone) / extract of 1 mL was added. After the addition of calcium to the sample, the OD was checked at 620 nm in a spectrophotometer (UV 1800, Shimadzu Corporation, Japan), on each 15 s over 5 min, and then every 1 min over 10 min.

All the experiments were done in triplicate. The final solutions were seen under a light microscope to analyze the density of formed crystals in the solution (Olympus, USA). Within the presence of CMME or Cystone percent, inhibition was estimated with control by the following formula.

The percentage of inhibition was calculated as: 1-Tsi/Tsc ×100

Where Tsc is the control (turbidity slope) and Tsi in the presence of inhibitor (turbidity slope) [19].

***In-vivo* Evaluation of the Antilithiatic Activity:**

Sodium Oxalate Induced Urolithiasis Model: The induced urolithiasis model by sodium oxalate was to investigate the antilithiatic effect of CMME in Wistar albino rats. This is an acute model of urolithiasis for 7 days. Sodium oxalate administration (0.07 g/kg, *i.p*) for 7 days induces lithiasis, and the anti-urolithiatic effect of leaf

extract (100 & 200 mg/kg, *p.o*) is evaluated by administering it for 1-7 days and compared with standard cystone (0.5 g/kg, *p.o*) 1-7 days. Group-I as Normal vehicle control received (0.5% gum acacia). Group II kept as diseases control group given sodium oxalate (0.07 g/kg, *i.p)* in distilled water for 1-7 days, Group-III & IV are test groups received CMME (100 mg/kg, *p.o*) & CMME (200 mg/kg, *p.o*) with sodium oxalate (0.07 g/kg, *i.p)* for 1-7 days in distilled water, and Group-V is standard reference group that received Cystone(0.5 g/kg, *p.o*) for 1to 7 days.

All the animals in different groups with water placed separately in metabolic cages for about 24 h, analyzed for physical parameters, urine volume, and urine pH on 0^{th} and 7^{th} day. To the urine, add a drop of the conc. HCl and stored at 40 ºC.

Under anesthesia blood withdrawn from retro on 0^{th} and 7^{th} day and sample centrifuged at 3000 rates for 15 min. Serum obtained was analyzed for creatinine, BUN, uric acid, calcium, phosphate, oxalate, sodium, and potassium [20-21].

Histopathology of Kidney: The kidney samples were mounted in 10% formalin for a min of 24 hours for the histopathological study. Paraffin pigment was prepared and cut to 5-µm sections by a rotary microtome. The kidney tissues were stained by Haematoxylin eosin dye. Slides were prepared beneath plane-polarized light and captured by the camera. Histopathological changes, calcium oxalate crystals within the urinary organ tissues were recorded [22].

Statistical Analysis: Data results as the mean ± SEM and analyzed by unidirectional analysis of variance followed by Dunnett's multiple comparison tests. A value of $p<0.05$ was thought of as statistically vital. We used graph pad prism for statistical analysis.

RESULTS: Methanolic *Citrus medica* leaf extract was explored for its anti lithiasis activity using suitable models *in-vitro* & *in-vivo*; all the results obtained in the study were included below.

Preliminary Phytochemical Analysis: Methanolic extract of *Citrus medica* disclosed tannins, terpenoids, alkaloids, glycosides, carbohydrates, saponins, and steroids presence.

Acute Toxicity Study: Administration of CMME 2000 mg/kg dose in no mortality or evidence of adverse effects implying that *Citrus medica* is nontoxic. No changes were observed in clinical signs, body weight, and behavioral patterns of mice throughout the 14 days study. Up to a 2000 mg/kg dose, *Citrus medica* was considered safe to be used.

***In-vitro* Evaluation of the Antilithiatic Activity:**

Nucleation and Aggregation Assay: The *in-vitro* antilithiatic activity of the methanolic extract of *Citrus medica* was done by using nucleation & aggregation assay shown in **Table 1** and **Fig. 1**. The blank group showed high turbidity, so the percent inhibition was known to be 0%. CMME has shown an increase in percent inhibition, a decrease in turbidity with an increase in dose, CMME 100-21.51%, and CMME 200-30.37%. The ability of the extract was compared to that of standard Cystone, and the percent inhibition value was showed to be 59.49.

TABLE 1: EFFECT OF CMME AND CYSTONE ON TURBIDITY AND PERCENTAGE INHIBITION IN *IN-VITRO* NUCLEATION AND AGGREGATION ASSAY METHOD

Group	Turbidity	Percentage Inhibition (%)
Blank	0.79	0
CMME 100 mg	0.62	21.51
CMME 200 mg	0.55	30.37
Cystone 500 mg	0.32	59.49

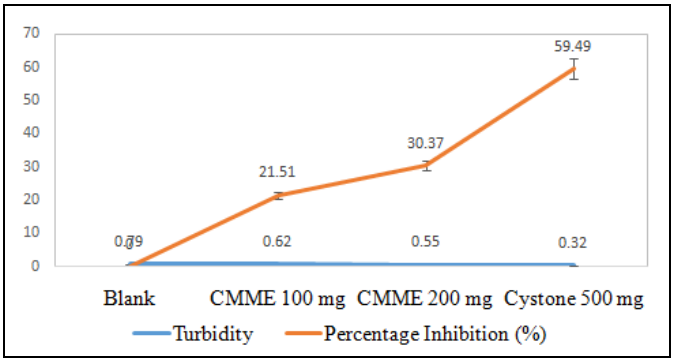

FIG. 1: EFFECT OF CMME AND STANDARD IN *IN-VITRO* NUCLEATION AND AGGREGATION ASSAY METHOD

Comparison Estimation of Microscopic Crystal Area in Different Groups: There is a significant decrease in Crystal count in cystone 500 mg, CMME 100 mg, and CMME 200 mg compared to the control group. Decrease crystal count is in following order control group-CMME 100 mg-CMME 200 mg-Cystone 500 mg. A is the control group, B is CMME (100 mg), C is CMME (200 mg), and D is Cystone (500 mg) shown in **Fig. 2**.

***In-vivo* Evaluation of the Antilithiatic Activity:**

Sodium Oxalate Induced Lithiasis Model: In the lithiasis disease control group, the creatinine, uric acid, phosphate, BUN, calcium, and potassium levels were increased, and sodium level was decreased after the administration of sodium oxalate. It was known to be significant in comparison with normal control. In treatment groups, the CMME, *i.e.*, 100 and 200 mg/kg, produced a significant increase in sodium levels and a significant decrease in creatinine, uric acid, phosphate, BUN, calcium, and potassium levels, respectively, and showed results compared with the disease control group. The activity of 200 mg/kg was shown a better response than the 100 mg/kg dose, and it was comparable to standard Cystone shown in **Table 2**, **Fig. 2**.

TABLE 2: EFFECT OF METHANOLIC EXTRACT OF *CITRUS MEDICA* ON SERUM LEVELS IN SODIUM OXALATE ON 7TH DAY

	Control	Disease control	CMME 100 mg/kg	CMME 200 mg/kg	Cystone 500 mg/kg
Creatinine (mg/dL)	0.77±0.02	2.72 ± 0.09^{aA}	$2.11\pm0.03^{a*A}$	$1.52\pm0.13^{a*ns}$	$1.47\pm0.02^{a*}$
Uric acid (mg/dL)	0.71±0.10	$2..74\pm0.06^{a}$	$1.89\pm0.10^{a*C}$	$1.65\pm0.09^{a*ns}$	$1.46\pm0.07^{a*}$
Phosphate (mg/dL)	3.16±0.10	6.53 ± 0.16^{a}	$5.33\pm0.13^{a*C}$	$4.76\pm0.12^{a*ns}$	$4.59\pm0.11^{a*}$
Potassium (mEq/L)	4.15±0.18	10.40 ± 0.52^{a}	$8.08\pm0.30^{a**D}$	$6.81\pm0.29^{a*ns}$	$6.73\pm0.30^{a*}$
BUN (mg/dL)	35.83±0.76	90.10 ± 0.92^{a}	$72.90\pm0.80^{a*A}$	$50.10\pm0.61^{a*ns}$	$48.98\pm0.84^{a*}$
Sodium (mEq/L)	142.58±0.55	93.38 ± 0.42^{a}	$116.82\pm0.30^{a*A}$	$127.10\pm0.28^{a*B}$	$129.51\pm0.17^{a*}$
Calcium (mg/dL)	9.37±0.39	13.43 ± 0.41^{a}	$12.48\pm0.18^{a\ ns}$	$12.2\pm0.10^{a***ns}$	$11.9\pm0.21^{a**}$

The values are expressed as mean ± SEM (n=6) analysis was performed with one way ANOVA followed by Dunnett's multiple comparison test against control (a=p<0.0001), against disease (*=p<0.0001, **=p<0.005, ***=p<0.05) and against cystone 500 (A=p<0.0001, B=p<0.001, C=p<0.01 and D=p<0.05). ns = Non-significant.

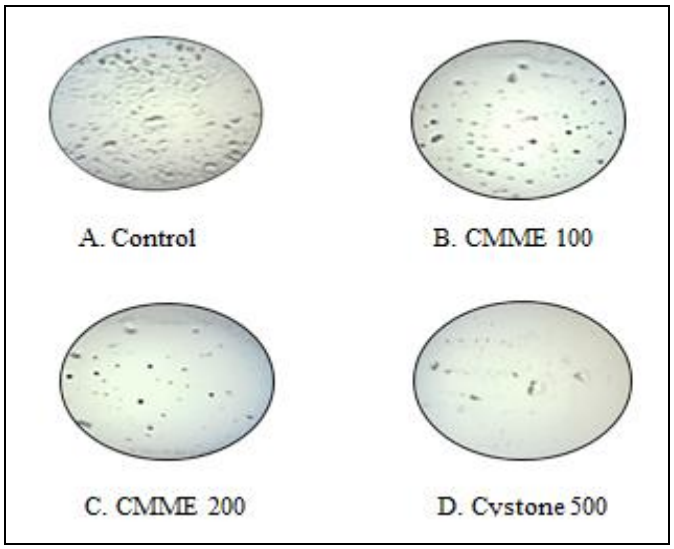

FIG. 2: COMPARATIVE ESTIMATION OF MICROSCOPIC CRYSTAL AREA BY *IN-VITRO* NUCLEATION AND AGGREGATION ASSAY METHOD

In the disease group, after sodium oxalate administration induces lithiasis; there is a decrease in urine pH (3.33±0.21), urine volume (4.6±0.15), and increased kidney weight (1.73±0.07), which is due to kidney impairment. CMME 100, CMME 200, and Cystone group increased the urine pH (5.5 ±0.22, 6.83±0.30, and 7.16±0.30), urine volume (5.3±0.08, 6.4±0.9, and 6.6±0.16), and decreased kidney weight (1.41±0.02, 1.27±0.01 and 1.13± 0.02) which is due to renal improvement.

The percent change in body weight significantly low in Sodium Oxalate lithiatic group (-4.33±0.65) was as there is an increase in body weight in CMME 100, CMME 200, Cystone, and control group (2.23±0.33, 3.89±0.90, and 4±0.25) shown in **Table 3**.

TABLE 3: EFFECT OF METHANOLIC EXTRACT OF *CITRUS MEDICA* ON FOLLOWING PHYSICAL PARAMETERS IN SODIUM OXALATE

Group	Percent change in bd.wt (gm)	Urine volume (mL)	Wet Kidney wt (g)	Dry kidney wt (g)	Urine pH
Control	4.83±0.40	8.1±0.10	1.06±0.01	0.45±0.03	6.5±0.22
Disease control	-4.33 ± 0.65^{a}	4.6 ± 0.15^{a}	1.73 ± 0.07^{a}	1.10 ± 0.08^{a}	3.33 ± 0.21^{a}
CMME 100 mg/kg	$2.23\pm0.33^{c*ns}$	$5.3\pm0.08^{b**B}$	$1.41\pm0.02^{a*A}$	$0.67\pm0.02^{b*C}$	$5.5\pm0.22^{c*B}$
CMME 200 mg/kg	$3.89\pm0.90^{*Cns}$	$6.4\pm0.9^{***ns}$	$1.27\pm0.01^{b*C}$	$0.53\pm0.013^{*ns}$	$6.83\pm0.30^{*ns}$
Cystone500 mg/kg	$4\pm0.25^{*ns}$	$6.6\pm0.16^{**ns}$	$1.13\pm0.02^{*ns}$	$0.52\pm0.01^{*ns}$	$7.16\pm0.30^{*ns}$

The values are expressed as mean ± SEM (n=6) analysis was performed with one way ANOVA followed by Dunnett's multiple comparison test against control (a=p<0.0001, b=p<0.005,c=p<0.05), against disease (*=p<0.0001,**=p<0.01, ***p<0.05) and against cystone 500 (A=p<0.0001, B=p<0.01,C=p<0.05). ns = Non-significant.

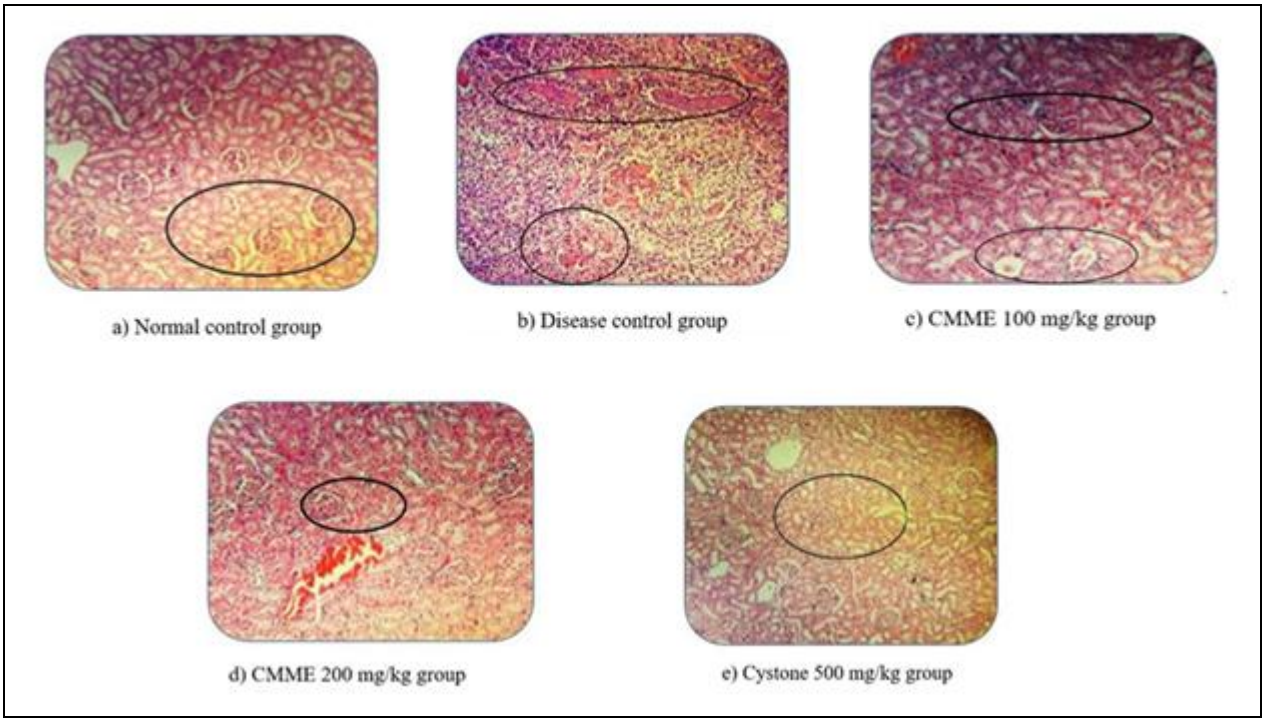

FIG. 3: HISTOPATHOLOGICAL ANALYSIS OF RENAL TUBULE AND GLOMERULI FOR SODIUM OXALATE INDUCED LITHIASIS MODEL FOR RAT

Histopathology Studies: Histopathology study of rat kidney in sodium oxalate induced lithiasis model. The pathological changes were viewed under a light microscope after staining with haematoxylin and eosin. The Control group resulted in normal healthy glomerulus and tubular cells without any haemorrhage or calcium oxalate deposition. The disease control group showed there was a moderate deposition of calcium oxalate crystals along with marked tubular elongation with inflammation in the tubular region, and glomerular degeneration is noted. CMME 100 mg/kg showed recovery of elongation of tubular cells in the kidney and slight degeneration of glomeruli observed. CMME 200 mg/kg showed a healthy glomerulus without any haemorrhage in the tubule and glomeruli. The tubular degeneration or inflammation was also found to be mild. Standard cystone 500 mg/kg showed normal glomeruli and tubule cells without any deposition or infiltration of calcium oxalate crystals shown in **Fig. 3**.

DISCUSSION: Urolithiasis is the condition where urinary calculi are formed or placed anywhere within the system aurogenitale, or the method of formation of stones within the excretory organ, bladder, and ureters (urinary tract) [23]. Cystone is beneficial in urolithiasis by disintegrating stones and corrects the crystal and colloid balance. It's been reported [24]. It is reported that flavonoids, terpenoids, steroids, saponins from different plants showed antilithiatic activity [5].

In calcium oxalate crystallization study by nucleation & aggregation assay, the process of nucleation & aggregation was studied in a sodium acetate buffer of pH 5.7 to simulate the conditions of urine to favour the process. Within the crystallization study, the cloudiness increased linearly up to 5 min, *i.e.*, nucleation, and so attenuated linearly up to fifteen min aggregation after the addition of $CaCl_2$ dihydrate. Administration of CMME and cystone along with calcium chloride dihydrate smothered the nucleation & aggregation process of CaC_2O_4 crystallization; decrease in nucleation in both phenomena showed percentage inhibition. The inhibition of *in-vitro* crystallization of CaC_2O_4 suggests that CMME has an influence on the formation of crystals from sodium oxalate and calcium chloride or their aggregation. Comparative estimation of microscopic crystal area by *in-vitro* nucleation & aggregation assay method in CMME and Cystone and the blank was studied. There we observed a significant decrease in crystal count in Cystone, CMME compared to blank. CMME 200 showed less calcium oxalate crystals compared to CMME 100.

Models used for *in-vivo* lithiasis induction are sodium oxalate, which causes calcium oxalate urolithiasis in excretory organ tubules of Wistar rats. Intraperitoneal administration of sodium oxalate to Wistar rats results in hyperoxaluria. The mechanism of sodium oxalate evoked stone formation is associated with an increase in the urinary oxalate levels due to poor solubility. Heterogeneous nucleation with inflicting calcium oxalate crystals aggregation in the renal tubules and epithelial cells damage was noted with high calcium oxalate crystals and oxalate levels, especially in nephron [20]. It has been evaluated that oxalate plays a crucial role in stone formation and vital risk factors in the pathologic process of renal stone [25].

Furthermore, the accumulation of the CaC_2O_4 crystals within the excretory organ enhanced the urinary pH that is one of the indications of urolithiasis [21]. In the $Na_2C_2O_4$ disease group, there is a decrease in urine pH, urine volume, increased kidney weight, which is due to renal impairment, CMME, and Cystone group results are *vice versa*. The percent change in body weight is significantly low in the $Na_2C_2O_4$ disease group as there is increasing order of CMME 100, CMME 200, Cystone, and control group. In the prior studies, $Na_2C_2O_4$ induction caused excessive elevation of uric acid, creatinine, and urea in the serum. GFR is decreased by the presence of stone in the excretory organ, which obstructs urine outflow in urolithiasis. It results in the building of waste products in the blood. The disease control group showed enhanced levels of urea, creatinine, uric acid, calcium, phosphate, electrolyte potassium, and decreased sodium levels. Cystone and CMME were found to show good nephroprotective activity by decreasing the increased levels of urea, creatinine, uric acid, calcium, phosphate, electrolyte potassium, and increased sodium electrolyte level in serum. CMME 200 showed better effects than CMME 100. Throughout the study control group remained normal.

During the histopathological examination of the kidney sections derived from the sodium oxalate model after the 7^{th} -day study, varying amounts of glomeruli were seen in the kidneys of experimental animal tissue. Disease group elicited in elongation of tubules with acute nephritis in glomeruli and high infiltration with interstitial inflammation. CMME 100 group showed degeneration of glomeruli and elongation of the tubule. Co-treatment with the CMME 200 and Cystone resulted in the normal glomerulus, but Cystone treated group there we observed slight elongation of the tubule. Essential oils present in CMME extract showed the antilithiatic property. Overall results explain that CMME has proven nephroprotective activity by sodium oxalate, *i.e.*, by controlling the renal stone formation and increasing urine flow and reduction in impaired renal tubules.

CONCLUSION: The findings of the present study highlight the ability of methanolic leaf extract of *Citrus medica* to prevent nucleation and aggregation growth of calcium oxalate crystals as proved in *in-vitro* studies. CMME has shown an increase in percent inhibition, decrease in turbidity with an increase in dose. The methanolic leaf extract *of Citrus medica* has decreased renal crystals in the sodium oxalate model. Though leaf extract of *Citrus medica* has shown both Phytoconstituents like properties, *i.e.*; ability to prevent stone formation and dissolved already formed stones, the preventive effect was more predominant. Preliminary screening of phytochemical CMME showed the presence of carbohydrates, alkaloids, terpenoids, glycosides, steroids, saponins, and flavonoids. The terpenoids, flavonoids, and saponins presence might be responsible for anti-lithiatic activity. This study encourages the isolation of active constituents of *Citrus medica* responsible for antilithiatic activity.

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CONFLICTS OF INTEREST: All authors have no conflicts of interest to declare.

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