Explor Anim Med Res.
Vol. 14, Issue 1, 2024
DOI: 10.52635/eamr/14.1.124-136

ISSN 2277-470X (Print), ISSN 2319-247X (Online)
Website: www.animalmedicalresearch.org

Research Article

ANTI-UROLITHIASIS AND DIURETIC ACTIVITIES OF *GREWIA FLAVESCENS* ROOTS

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Received 10 December 2023, revised 16 June 2024

ABSTRACT: The purpose of the present study is to investigate the effect of aqueous and methanolic extracts of *Grewia flavescens* (AEGF and MEGF respectively) on kidney stones using *in vitro* and *in vivo* methods. *In vitro* anti-urolithiasis activity was performed by different assays like nucleation, crystal growth, crystal aggregation, and crystal dissolution assays were performed and cell viability studies were performed on the MDCK cell line before and after exposure to oxalate. An HPTLC and HPLC method was developed for the quantification of diosgenin, catechin, rutin, gallic acid, and quercetin in extract. For an *in vivo* study, performed a diuretic activity model and sodium oxalate-induced urolithiasis in male Wistar albino rats. Determination of body and kidney weight, measurement of various biochemical parameters in biological samples, and examination of histology of the kidney at the end of the experiment were also done. AEGF and MEGF exhibited a concentration-dependent inhibitory activity on nucleation and aggregation along with a decreased number of calcium oxalate crystals produced in calcium oxalate metastable solutions in the *in vitro* experiments. Cell line study showed that extracts showed cell viability in the presence of oxalate. Co-administration of AEGF and MEGF with sodium oxalate has significantly ($p<0.001$) increased the urine volume and the level of calculus inhibitors and decreased the level of calculus promoters. HPTLC and HPLC data showed that extracts contain catechin, rutin, gallic acid, and quercetin. These results data indicate that AEGF and MEGF showed significant activity in urolithiasis which might be due to their diuretic, crystal formation inhibitory effects and their ability to increase the levels of inhibitors and decrease the level of promoters of urolithiasis due to the presence of active constituents in extracts.

Keywords: Diuresis, Kidney stone, *Grewia flavescens*, Sodium oxalate.

INTRODUCTION

Urolithiasis, sometimes known as kidney stone disease, is a painful condition that commonly occurs in the kidney and/or urinary system. It was originally discovered in Egyptian mummies in approximately 4800 BCE and was categorized as the third most common disorder in the category of urinary disorders. It may lead to renal calcification and has a major impact on public health [1, 2, 3]. About 4 to 15% of the population globally suffer from urinary stones. According to a recent study, the incidence of urolithiasis increased by 48.57% worldwide between 1990 and 2019 [4]. Renal kidney stones affected 7.1% of women and 10.6% of men in the United States in 2012, according to the National Health and Nutrition Examination study [5]. Men are more affected than women, and the condition is more common in both sexes between the ages of 20 and 49, according to

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epidemiological studies [6]. A recurrence rate of 10-23% occurs annually, with 50% of cases occurring in 5-10 years and 75% in 20 years. According to Sohgaura and Bigoniya [7], urolithiasis affects an estimated 12% of Indians annually, with the "Stone belt" states of Gujarat, Maharashtra, Rajasthan, Delhi, Punjab, and Haryana having the highest incidence rates. The multifaceted nature of urolithiasis renders its etiology intricate and highly variable.

The management of the ailment is significantly influenced by the size and placement of the stones. Most often, stones are removed surgically using techniques including extracorporeal shock wave lithotripsy, ureteroscopy, and percutaneous nephrolithotomy; but, regrettably, after surgical stone removal, recurrence of stones was observed in about half of the patients [8]. Aeckart and Schroder [9] state that complications from surgery include renal fibrosis, hemorrhage, tubular necrosis, and hypertension. Because urolithiasis is a chronic condition with frequent recurrence, there is increasing interest in alternative supportive therapies that use natural products and ayurvedic medicines to control the condition. It is said that Ayurvedic medicine uses a variety of conventional medicines to treat urolithiasis. Kieley *et al.* [10] state that without causing any appreciable side effects, these traditional herbs may display a wide range of complicated functions, such as diuretic, analgesic, antibacterial, anti-inflammatory, antispasmodic, litholytic, and anti-calcifying properties.

Tiliaceae plant *Grewia flavescens*, commonly referred to as "donkey's berry", is a tiny tree or straggling shrub that is mostly found in Bihar, Rajputana, the upper Gangetic plain, and central and southern India. According to a phytochemical analysis, *G. flavescens* ethanolic extract contains significant levels of flavonoids, glycosides, phenolics, proteins, tannins, triterpenoids, carbohydrates, saponins, and phytosterols [11]. In the West Kordofan region of Western Sudan, Africa, a decoction of *G. flavescens* roots, called locally abu halaf, is used to treat leprosy and stomach ailments [12]. The root of the plant, locally known as motsotsojane in Setswana and rough-leaved raisin in English, is boiled in milk and used to treat large sores under the chin [13]. Root powder and decoction of the plant, also known as Kali-Siali, is used locally in Rajasthan's Aravali regions. The goal of this study is to assess the effect of *G. flavescens*' AEGF and MEGF on experimentally induced calcium oxalate crystals using both *in vitro* and *in vivo* methods.

MATERIALS AND METHODS

Materials

The experiment employed only analytically graded substances. Standard medication Cystone (Himalaya Drug Company) was bought from Ahmedabad's local market. We purchased the Accucare kits (calcium, uric acid, urea, phosphorus, creatinine, and uric acid) from Lab-Care Diagnostics Pvt Ltd in Ahmedabad, Gujarat, India. The MDCK cell line was obtained from NCCS in Pune, India, while HiMedia in Mumbai, India provided the EZcount MTT cell assay kit, cell line media, culture, and reagents.

Plant material and preparation of plant extract

Early in August, fresh, dried *Grewia flavescens* roots were gathered from the Balaram-Ambaji and Aravali regions of Gujarat, India. The specimen was sent to the Department of Pharmacognosy once the roots were verified. An electric grinder was used to finely powder the dry roots. For later use, the powdered medication was kept at room temperature in a sealed container. For a full day, 500 ml of petroleum ether was refluxed with 100 grams of dried *G. flavescens* root powder. After gathering a layer of petroleum ether, the powder was refluxed once more for 24 hours with 500 ml of methanol. For aqueous extract, 100 gm of dried powder was refluxed with 500 ml water for 24 hr. Both extracts were filtered & evaporated using a rotary vacuum evaporator at 50 °C, which collected dry extract labeled as methanolic extract (7.15 % w/w) (MEGF) and aqueous extract (8.2 % w/w) (AEGF) respectively. Both extracts underwent phytochemical screening to determine the type of phyto-constituents and total flavonoid and saponin were determined using standard methods.

HPTLC and HPLC of Extracts

Using a mobile phase of ethyl acetate: toluene: formic acid: methanol (4:5:1.2:0.3), HPTLC of AEGF and MEGF were carried out. The plate was heated and then examined at 540 nm under a Camag UV chamber after being derivatized by spraying an anisaldehyde sulphuric acid solution.

Using a Hupersill C18 5.0 µ column and a gradient elution procedure lasting 30 minutes, the chemicals were separated using a chromatographic method. The mobile phase consisted of acetonitrile (A) and 0.5% v/v formic acid in water (B) at a flow rate of 1.0 mL/min. (At first, 0-5 minutes, 100% B; 5-10 minutes, 90% B; 10-15 minutes, 70% B; 15-20 minutes, 30% B; 20-22 minutes, 2% B; 22-30 minutes, 100% B).

PDS detectors were used to detect the presence of rutin and quercetin at 254 nm and catechin, diosgenin, and gallic acid at 285 nm. Determines the chemical peak in extracts and measures the metabolites using standard solution peak (retention time) [14].

In vitro experiments

Cell line study

The Madin Darby canine kidney (MDCK) epithelium-derived cell line was acquired from NCCS in Pune, India. The MDCK cell line was cultured in a 75 cm^2 tissue culture flask with 5% CO_2 at 37°C and a sub-confluent monolayer. The cell line was consistently kept in a 1% antibiotic-antimycotic solution and 10% fetal calf serum in Dulbecco's Modified Eagle's Medium (DMEM).

Cytotoxicity study of aqueous and methanolic extracts

For cytotoxicity assay, MDCK cells were treated with 100 µl of various concentrations of both extracts (0.05, 0.5, 5, and 50 µg/ml) for a further 48 hrs in a serum-free medium. Using MTT cell assay kits cytotoxicity of EAFGF was determined.

Oxalate-induced cell injury on MDCK cell line

80% confluence cells were incubated in DMEM medium containing 1 mM NaOx in the presence of various concentrations of AEGF and MEGF (0.05, 0.5, 5, and 50 µg/ml) for a further 48 hrs. Injury of NaOx on MDCK cells with both extracts and standard drug was assessed by measuring cell viability using the MTT assay kit method and monitoring LDH leakage into the medium.

In vitro Nucleation assay, Aggregation assay, Crystal growth, and crystal dissolution assay were formed as described by our previous article using different concentration ranges of AEGF and MEGF [2].

Animal study

The Institutional Animal Ethics Committee of the Institute of Pharmacy gave its approval to the study protocol. (IP/PCOG/PHD/19/015, dated 28 July 2016) In a controlled environment (temperature of 22-25°C, relative humidity (55-5%), and 12-hour light-dark cycle), adult male Wistar albino rats (weighing 180-250g) were housed at the Institute of Pharmacy, Nirma University, Ahmedabad, Gujarat, India. Animals received food pellets and water ad libitum.

Acute toxicity study

The acute toxicity study was carried out in Wistar male rats as per the guidelines by the OECD. Zero mortality was observed up to an extract dose of 6000 mg/kg.

Determination of diuretic activity

Six groups of six healthy rats were created using a random number generator. Saline (20 ml/kg) and hydrochlorothiazide (10 mg/kg, as standard) were given to the normal and standard groups, respectively, while treated groups received the same ml of saline containing 400 mg/kg and 800 mg/kg of AEGF and MEGF, p.o. as a single dose. The urine was collected in cylinders at an interval of one hour for 6 hr and urine volume was measured [15].

Sodium oxalate model of urolithiasis

Seven groups of six rats each (normal, disease control, standard, and four treatment groups with two doses of AEGF and two doses of MEGF). Saline (2.5 ml/kg) and 70 mg/kg sodium oxalate in saline were given intraperitoneally for 7 days to all animals except those in the normal group (Group I) to develop urolithiasis. Cystone (750 mg/kg, p.o.) was given to the animals in the control group (Group III) for 7 days, along with doses of AEGF (400 mg/kg and 800 mg/kg, respectively) and MEGF (400 mg/kg and 800 mg/kg, respectively) for Groups IV, V, VI and VII. Animal Body weights were recorded during the experiment and relative body weights (RBW) of animals were calculated.

Urine and serum collection and analysis

On the 8th day, animals were kept in individual metabolic cages and 24 urine samples were collected. The urine volume, urinary pH, crystalluria in urine, and total calcium, uric acid, magnesium, urea, and phosphate levels were measured in urine using standard kits. The citrate and oxalate were estimated by the method described by Rajagopal [16] and Hodgkinson [17] respectively. Blood was collected under light anesthetic conditions from the retro-orbital plexus by capillary method and separated serum was used to analysis of calcium, magnesium, uric acid, creatinine, and blood urea nitrogen (BUN) using diagnostic kits.

Kidney histopathology and homogenate analysis

The abdomen was incised and opened and both kidneys were removed from each animal under study. Extraneous tissue was removed from isolated kidneys and one kidney was fixed with 10% v/v neutral formalin solution and sent to histology services for Hematoxylin and Eosin staining. The section of the

kidney was observed under a microscope for examination of calcium oxalate crystal depositions and the presence of glomerular congestion, tubular casts, epithelial adhesion, blood vessel congestion, interstitial edema, and inflammatory cells. Another kidney was finely chopped and 20% of the homogenate was prepared in Tris-HCl buffer (pH 7.4). Kidney homogenate was used for the determination of calcium, uric acid, phosphate, oxalate, urea, LDH, and catalase.

Statistical analysis

Results data were expressed as mean ± SEM. The results among the groups were analyzed by one-way ANOVA followed by Dunnett's test using GraphPad Prism version 6. Results were considered significant when the value of $p<0.05$ or $p<0.001$.

RESULTS AND DISCUSSION

Phytochemical screening and quantitative estimation of phytoconstituents

Chemical tests were used to qualitatively analyze the AEGF and MEGF for different phytoconstituents. The results of the investigation showed the existence of phenolic compounds, carbohydrates, phytosterols, and flavonoids. It was discovered that 3.91 ± 0.34 and 5.54 ± 0.2mg quercetin equivalents/g of extract comprised AEGF and MEGF's total flavonoid respectively. The total saponin content of AEGF and MEGF were found 43.04 ± 0.85 and 29.40 ± 0.99 mg diosgenin equivalent/g of extract respectively. These phytoconstituents have essentially little impact in preventing the formation of urinary stones. It is well known that saponins have antilithic properties and degrade mucoproteins, which are crucial components of the stone matrix [18]. Flavonoids with strong antioxidant and anti-lithiatic properties include rutin, quercetin, hyperoside, and diosmin [19].

HPTLC and HPLC data of AEGF and MEGF

Fig. 1 showed the spot of standard compound (Gallic acid, Rutin, Catechin, Quercetin, Diosgenin), AEGF, and MEGF after derivation with anisaldehyde sulphuric acid which showed that AEGF and MEGF presence of Rutin, Catechin, Gallic acid, and Quercetin. Fig 2a and 2b showed a densitogram of AEGF and MEGF at 254 nm, respectively.

HPLC chromatogram showed that rutin and quercetin retention time at 12.88 and 15.75 respectively at 254 nm wavelength, while at 285 nm wavelength gallic acid, diosgenin, and catechin showed retention time at 8.24, 11.05, and 11.80 respectively. Fig. 3 shows the standard HPLC chromatogram of Rutin, Quercetin, Gallic acid, Diosgenin, and Catechin and Fig. 4 and Fig. 5 shows the AEGF and MEGF chromatogram which indicate that both extracts contain Gallic acid, Rutin, and Quercetin and catechin.

***In vitro* study**

MDCK cell line study

MDCK cells are a common model system for the distal/collecting duct in the pathogenesis of renal disease. They are a normal kidney epithelial cell line from the distal tubules [20]. A cytotoxicity study was performed on the MDCK cell line for AEGF, MEGF, Cystone, and oxalate. After 24 hrs of exposure to AEGF, MEGF, Cystone and oxalate on MDCK cells showed 77.80±1.01, 82.57±0.39, 79.37±0.64 and 15.51±0.69 % at higher concentrations (5 µg/ml) in AEGF, MEGF, Cystone, and oxalate respectively. While oxalate has very low cell viability 15.51±0.69 % at higher concentrations (5 µg/ml) (Table 1).

On exposure of oxalate to the MDCK cell line, oxalate produced cytotoxicity up to 90% means only 10 % cell viability, whereas in the presence of AEGF, MEGF, and cystone showed 68.41±0.53 %, 70.28±0.49 % and 50.44±0.59 % at higher concentration (5 µg/ml), respectively. (Table 2) AEGF, MEGF, and cystone have shown 92.50 ± 0.77%, 78.55 ± 0.49%, and 53.92 ± 0.58% at higher concentrations (5 µg/ml) respectively. Results showed that AEGF, MEGF, and cystone showed a protective effect on MDCK cell lines. The above results indicated that exposure to oxalate reduced the cell viability and increased the LDH release. AEGF and MEGF increased the cell viability and reduced

Table 1. Percentage cell viability after exposure of extracts, cystone and oxalate on MDCK cell.

Concentration	% Cell viability			
	Oxalate	Cystone	AEGF	MEGF
0.05 µg/ml	33.33±0.60	88.39±0.57	90.62±0.96	90.75±0.56
0.5 µg/ml	27.62±1.38	84.62±1.24	87.58±0.87	89.49±0.49
5 µg/ml	18.08±1.06	80.50±0.80	78.07±0.66	86.23±0.69
50 µg/ml	15.51±0.69	79.37±0.64	77.80±1.01	82.57±0.39

Table 2. Percentage cell viability of cells in presence of AEGF, MEGF and cystone after exposure of oxalate on MDCK cells.

Concentration	% Cell viability		
	Cystone	AEGF	MEGF
0.05 µg/ml	39.93±0.80	54.75±0.49	58.19±0.20
0.5 µg/ml	41.39±0.59	59.45±0.54	61.87±0.90
5 µg/ml	44.63±0.40	64.71±0.65	66.54±0.43
50 µg/ml	50.44±0.59	68.41±0.53	70.28±0.49

LDH release which indicated that both extracts protect renal epithelial cells from crystal damage, stop crystals from adhering to cell lines, reduce the production of free radicals, and lower the release of LDH.

In vitro nucleation, crystal growth, crystal aggregation, and crystal dissolution study

Renal stone formation is caused by nucleation, which is a crucial step in the crystallization process and results in crystal growth and aggregation. Substances that inhibit crystallization, regulate these processes, or reduce oxalate supersaturation are the mainstays of treatment options for renal stones. Numerous inhibitors can influence the nucleation, development, or aggregation of crystals [21]. The *in vitro* assays used in this work were created to evaluate important components including crystal nucleation, growth, and aggregation.

According to Park *et al.* [22], urine oxalate levels are closely correlated with the severity of stone formation because supersaturation levels of ions induce particles to spontaneously crystallize, which increases the nucleation rate. As oxalate concentrations increased, the nucleation rate rose. Tables 3, and 4 demonstrate that AEGF and MEGF exhibited the highest level of inhibition at a larger dose of 1500 µg/ml at a lower concentration of NaOx (2 mM), which was more effective than that of cystone (Table 5), data also indicated that as the concentrations of oxalate increased the inhibition rate also decreased as compared to the lower concentration of oxalate ions. Inhibition of Nucleation may be the ability of extracts to interact with oxalate and calcium ions in solution and reduce the super saturation.

After nucleation, the fluid's unbound calcium and oxalate particles attach to the formed CaOX crystal and cause it to grow. The concentrations of calcium and oxalate have an impact on the formation of crystals. The current investigation demonstrated that

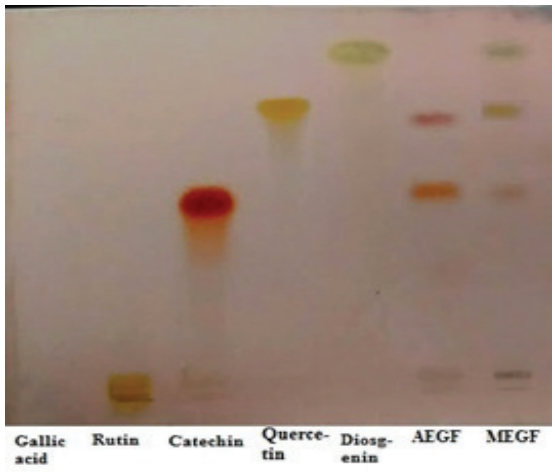

Fig. 1. Chromatograms of AEGF, MEGF and standard quercetin, rutin, gallic acid, catechin and diosgenin. [Visualization was under UV light of wavelength 254 nm after derivatization by anisaldehyde sulphuric acid solution].

AEGF and MEGF inhibit crystal development in a dose-dependent way; as shown in Table 6 and 7, respectively; however, the inhibition rate was shown to be lower at the same fractional dose when the concentration of sodium oxalate gradually increased from 2 mmol/ml to 3.5 mmol/ml. Table 8 showed that cystone drugs also had a similar manner effect but lower as compared to AEGF and MEGF.

Small crystals are readily removed from the urine, but large crystals are formed when many crystals unite and adhere to one another. These large crystals are usually maintained in the renal tubules and play a role in the development of kidney stones. Thus, it is thought that a critical step in the development of renal stones is the crystal aggregation process. Table 9, shows that AEGF, MEGF, and cystone suppressed the aggregation in the solution and prevented the large crystal formation in a dose-dependent manner.

Some studies suggest that chemical therapy could assist, in improving the efficacy of stone formation by dissolving tougher or larger stones. The current study demonstrated that AEGF and MEGF were effective in increasing the dissolution of calcium oxalate stones in a concentration-dependent manner, suggesting that both extracts include some of the components involved in this process. (Table 10).

Effect on dieresis

AEGF showed significant diuretic activity at the dose of 400 and 800 mg/kg (10.96±0.06 and 15.30±0.14 ml/100 gm/6 hr, respectively) and MEGF showed (11.32±0.28 and 16.06 ± 0.09 ml/100 gm/6

Table 3. Effect of AEGF on calcium nucleation with the increasing amount of sodium oxalate.

Conc. of drug (µg/ml)	% Inhibition of nucleation				
	2 mmol NaOx	4 mmol NaOx	6 mmol NaOx	8 mmol NaOx	10 mmol NaOx
250	60.14±0.41	58.39±0.44	55.41±0.29	54.05±0.20	53.57±0.25
500	68.44±0.72	66.60±0.21	63.09±0.21	62.42±0.22	61.89±0.22
750	74.25±0.51	72.20±0.38	69.50±0.38	67.41±0.21	66.55±0.23
1000	78.17±0.52	76.72±0.21	72.93±0.21	71.16±0.22	70.22±0.21
1250	80.78±0.61	78.43±0.33	75.67±0.29	73.36±0.21	72.81±0.23
1500	82.68±0.92	81.30±0.32	79.29±0.25	75.24±0.22	74.54±0.36

Table 4. Effect of MEGF on calcium nucleation with the increasing amount of sodium oxalate.

Conc. of drug (µg/ml)	% Inhibition of nucleation				
	2 mmol NaOx	4 mmol NaOx	6 mmol NaOx	8 mmol NaOx	10 mmol NaOx
250	54.92±0.83	54.00±0.44	53.25±0.46	52.35±0.29	50.98±0.32
500	63.58±0.72	61.96±0.56	60.59±0.38	59.84±0.29	58.82±0.25
750	69.04±0.61	68.12±0.58	65.93±0.39	64.87±0.30	64.18±0.30
1000	73.07±0.83	72.32±0.44	69.94±0.34	68.95±0.22	68.00±0.26
1250	76.63±0.62	75.89±0.28	73.13±0.25	72.32±0.36	71.81±0.25
1500	78.41±0.72	77.35±0.44	75.33±0.34	74.03±0.36	73.73±0.16

Table 5. Effect of cystone on calcium nucleation with the increasing amount of sodium oxalate.

Conc. of drug (µg/ml)	% Inhibition of nucleation				
	2 mmol NaOx	4 mmol NaOx	6 mmol NaOx	8 mmol NaOx	10 mmol NaOx
250	37.93±0.44	35.86±0.17	32.78±0.13	29.70±0.18	25.22±0.04
500	43.93±0.56	42.31±0.22	39.19±0.17	34.91±0.11	30.97±0.13
750	50.96±0.44	47.24±0.17	43.33±0.08	39.61±0.07	34.44±0.10
1000	54.79±0.45	51.96±0.18	46.82±0.17	42.18±0.15	37.86±0.11
1250	58.62±0.40	54.49±0.23	48.63±0.19	44.27±0.11	40.46±0.13
1500	61.56±0.34	56.95±0.17	50.00±0.17	45.59±0.12	41.48±0.13

Table 6. Effect of AEGF on calcium oxalate crystal growth with the increasing amount of sodium oxalate.

Conc. of drug (µg/ml)	% Inhibition of CaOX crystal growth			
	2 mmol NaOx	2.5 mmol NaOx	3 mmol NaOx	3.5 mmol NaOx
250	62.91±0.35	56.97±0.32	43.10±0.26	36.66±0.31
500	67.27±0.42	60.10±0.33	44.59±0.39	39.09±0.42
750	71.39±0.43	62.11±0.63	46.53±0.34	44.09±0.54
1000	76.52±0.65	67.71±0.31	48.47±0.32	47.01±0.25
1250	80.19±0.37	71.96±0.27	51.52±0.33	50.55±0.42
1500	83.12±0.56	75.09±0.45	54.51±0.31	53.40±0.30
1750	87.28±0.42	77.62±0.59	57.12±0.30	57.84±0.42
2000	90.87±0.49	80.90±0.26	59.35±0.29	62.01±0.41

Table 7. Effect of MEGF on calcium oxalate crystal growth with the increasing amount of sodium oxalate.

Conc. of drug (µg/ml)	% Inhibition of CaOX crystal growth			
	2 mmol NaOx	2.5 mmol NaOx	3 mmol NaOx	3.5 mmol NaOx
250	70.33±0.43	58.46±0.32	46.45±0.32	44.51±0.36
500	73.67±0.35	61.44±0.33	48.54±0.38	45.90±0.18
750	76.93±0.49	63.83±0.39	51.45±0.64	47.01±0.36
1000	79.95±0.42	67.63±0.31	54.28±0.52	49.58±0.55
1250	82.88±0.43	70.69±0.38	57.04±0.51	53.47±0.36
1500	85.16±0.35	73.22±0.45	60.32±0.58	55.90±0.42
1750	88.99±0.42	75.16±0.38	63.46±0.39	57.43±0.36
2000	93.15±0.43	77.77±0.58	65.99±0.51	59.72±0.37

Table 8. Effect of cystone on calcium oxalate crystal growth with the increasing amount of sodium oxalate.

Conc. of drug (µg/ml)	% Inhibition of CaOX crystal growth			
	2 mmol NaOx	2.5 mmol NaOx	3 mmol NaOx	3.5 mmol NaOx
250	54.93±0.49	51.15±0.39	31.76±0.38	29.09±0.42
500	58.92±0.42	54.21±0.45	36.53±0.39	31.18±0.40
750	62.83±0.41	58.16±0.46	42.35±0.45	35.13±0.41
1000	67.23±0.40	60.85±0.51	45.11±0.45	38.26±0.48
1250	69.43±0.56	65.02±0.32	49.14±0.58	41.11±0.30
1500	73.10±0.42	68.45±0.22	51.60±0.53	44.23±0.60
1750	77.09±0.49	72.85±0.39	54.51±0.45	46.73±0.25
2000	80.92±0.42	74.34±0.40	58.61±0.38	51.25±0.43

Table 9. Effect of AEGF, MEGF and cystone on crystal aggregation.

Conc. of Drug (µg/ml)	% Inhibition of crystal aggregation		
	AEGF	MEGF	Cystone
250	40.38±0.23	36.99±0.20	32.51±0.14
500	45.30±0.24	44.10±0.28	36.06±0.25
750	48.41±0.22	50.22±0.23	39.12±0.19
1000	52.35±0.23	52.95±0.19	42.02±0.23
1250	56.23±0.19	56.12±0.24	44.10±0.19
1500	58.30±0.24	59.51±0.19	47.32±0.23
1750	60.43±0.21	62.13±0.28	50.00±0.19

Table 10. Effect of AEGF, MEGF and cystone on crystal dissolution.

Conc. of Drug (µg/ml)	% Dissolution of CaOx crystals		
	AEGF	MEGF	Cystone
250	49.93±0.52	40.03±0.32	44.00±0.7
500	52.50±0.34	44.90±0.37	48.00±0.28
750	54.93±0.29	50.00±0.40	50.00±0.29
1000	57.43±0.28	54.93±0.46	55.00±0.86
1250	59.90±0.32	60.00±0.28	57.40±0.49
1500	64.73±0.42	64.93±0.34	59.86±0.41
1750	69.96±0.37	70.06±0.35	64.23±0.43
2000	74.93±0.35	83.03±0.52	69.56±1.09

hr, respectively) as compared to normal group (8.51 ± 0.26 ml/100 gm/6 hr), furthermore, the effect of MEGF and AEGF showed significant difference ($p< 0.05$) at a dose of 800 mg/kg when compared with the standard diuretic agent, furosemide (14.08 ± 0.39 ml/100 gm/6 hr) (Fig. 6).

Effect on various parameters of sodium oxalate model

Effect on the relative body and organ weight, urine volume, urinary pH, and crystalluria

Larger stones may obstruct the renal capillary tube and cause discomfort and pain, this condition may lead to a decrease in food consumption, which results in a decrease in body weight and an increase in kidney weight due to the deposition of crystals in the kidney and decreased the urine formation which promotes the super-saturation levels of ions and acidic urinary pH prevent the solubility of calcium oxalate stone. Result data shown in Table 11 indicated that administration of sodium oxalate in animals showed a significant ($p< 0.001$) decrease in relative body weight, urinary volume, and urinary pH, while relative organ weight significantly increased as compared to the normal group on the 7th day. In contrast to the disease control group concurrent administration of AEGF and MEGF (400 and 800 mg/kg) and cystone (750 mg/kg, standard medication) resulted in a significant ($p<0.001$) rise in relative body weight, increased urinary volume, and urinary pH and decrease in relative organ weight. This effect may be due to the presence of different phyto-constituents which give pain relief, diuretic activity which lowers the super-saturation levels of ions, and increased pH improves the stone solubility in urine. These crystal dissolution results were also supported by a crystalluria study in urine, which showed that a disease control group of animals, had more and larger crystals than the urine of the normal group [Fig. 7(a,b)]. Urine of animals treated with cystone and AEGF, MEGF revealed very few and tiny calcium oxalate crystals [Fig. 7(c,d,e,f,g)].

Effect on urolithiasis promoters and inhibitors

Several investigations showed that animals given sodium oxalate developed stones as a result of

Table 11. Effect of AEGF and MEGF on body weight and organ weight, urine volume, urinary pH in sodium oxalate induced urolithiasis.

	Relative body weight (%)	Relative organ weight (%)	Urine Volume (ml/24 hr)	Urinary pH
Group I (Normal control)	101.4±0.3	0.49±0.03	8.12± 0.42	6.76±0.04
Group II (Disease control)	96.16±0.51a*	0.61±0.03 a*	4.37±0.24 a*	5.32±0.04 a*
Group III (Standard control)	100.7±0.14b*	0.52±0.01 b#	11.00±0.21 b*	6.45±0.02 b*
Group IV (AEGF 400 mg/kg)	100.9±0.17 b*	0.52±0..02 b#	12.25±0.32 b*	6.05±0.06 b*
Group V (AEGF 800 mg/kg)	101.1±0.23 b*	0.48±0.01 b#	16.58±0.51 b*	6.52±0.04 b*
Group VI (MEGF 400 mg/kg)	101.5±0.18 b*	0.54±0.02 b#	11.88±0.14 b*	6.15±0.12 b*
Group VII (MEGF 800 mg/kg)	101.6±0.13 b*	0.49±0.01 b#	17.25±0.43 b*	6.50±0.08 b*

[All values are expressed in mean ± SEM (n = 6), one-way ANOVA followed by Dunnett's test; *p< 0:001 statistically significant, # p< 0:005 statistically significant, [a] = compared with normal Group, [b] = compared with disease control group].

Table 12. Effect of AEGF and MEGF on serum, urine and kidney homogenate parameters in sodium oxalate induced urolithiasis in rats.

	Group I (Normal Control)	Group II (Model Control)	Group III (Standard Control)	Group IV (AEGF 400 mg/kg)	Group V (AEGF 800 mg/kg)	Group VI (MEGF 400 mg/kg)	Group VI (MEGF 800 mg/kg)
Urine Parameter (mg/24 hr)							
Calcium	2.85±0.19	4.8±0.26a*	3.18±0.10 b*	3.81±0.09b*	2.96±0.08 b*	3.45±0.12b*	2.57±0.26 b*
Oxalate	4.14±0.17	10.15±0.05 a*	6.80±0.08 b*	7.14±0.07b*	5.57±0.08b*	7.61±0.15b*	5.43±0.13b*
Phosphate	4.22±0.11	6.95±0.27 a*	5.60±0.19 b*	5.78±0.16b*	4.93±0.27b*	5.98±0.15b*	4.93±0.16b*
Uric acid	1.93±0.06	3.89±0.07 a*	2.16±0.06 b*	2.64±0.13b*	2.08±0.06b*	2.51±0.10b*	1.91±0.13b*
Urea	0.58±0.03	1.36±0.08 a*	0.80±0.05 b*	0.96±0.05b*	0.96±0.06b*	0.92±0.03b*	0.82±0.02b*
Citrate	21.25±0.92	7.99±0.08 a*	18.46±0.18 b*	12.73±0.30b*	20.46±0.29b*	12.90±0.08b*	19.42±0.45b*
Magnesium	3.10±0.26	1.07±0.07 a*	2.83±0.11 b*	2.36±0.27b*	2.41±0.16b*	2.11±0.18b*	2.49±0.19b*
Creatinine Clearance	36.17±1.51	10.00±2.05 a*	44.61±2.01 b*	48.33±11.76b*	64.74±6.08b*	36.16±7.02b*	76.53±6.72b*
Serum Parameter (mg/dl)							
Calcium	9.71±0.19	12.26±0.16 a*	9.97±0.19 b*	10.90±0.14b*	10.16±0.13b*	10.46±0.14b*	9.99±0.20b*
Phosphate	4.63±0.51	7.90±0.23 a*	5.33±0.32 b*	5.59±0.27b*	4.57±0.23b*	5.13±0.25b*	4.29±0.32b*
Uric acid	3.68±0.40	6.28±0.18 a*	4.23±0.25 b*	3.64±0.24b*	2.96±0.13b*	3.85±0.17b*	2.88±0.14b*
Urea	12.50±1.03	27.68±0.89 a*	15.18±0.89 b*	19.64±1.03b*	9.82±0.89b*	16.96±2.2b*	10.71±1.45b*
Magnesium	3.30±0.11	1.82±0.08 a*	2.83±0.11 b*	2.63±0.16b*	2.99±0.10b*	2.57±0.09b*	3.17±0.08b*
Kidney Homogenate Parameter							
Calcium (mg/gm tissue)	4.92±0.08	8.14±0.11 a*	6.03±0.07 b*	6.59±0.07 b*	5.47±0.21b*	5.97±0.07b*	5.78±0.06b*
Oxalate (mg/gm tissue)	1.39±0.09	5.91±0.16 a*	2.41±0.31 b*	2.82±0.14b*	2.16±0.12b*	2.75±0.19b*	2.09±0.09b*
Phosphate (mg/gm tissue)	2.95±0.23	5.14±0.31 a*	3.41±0.13 b*	3.68±0.15b*	3.11±0.14b*	4.05±0.13b*	3.24±0.18b*
Catalase (nmoles of H_2O_2 utilized/ min/ mg Protein)	1.69±0.05	0.72±0.05 a*	1.39±0.11 b*	1.08±0.08b*	1.34±0.08b*	1.17±0.07b*	1.14±0.14b*

[All values are expressed in mean±SEM (n = 6), one-way ANOVA followed by Dunnett's test. * p < 0:001 statistically significant, # p < 0:005 statistically significant, a = compared with normal Group, b = compared with disease control group].

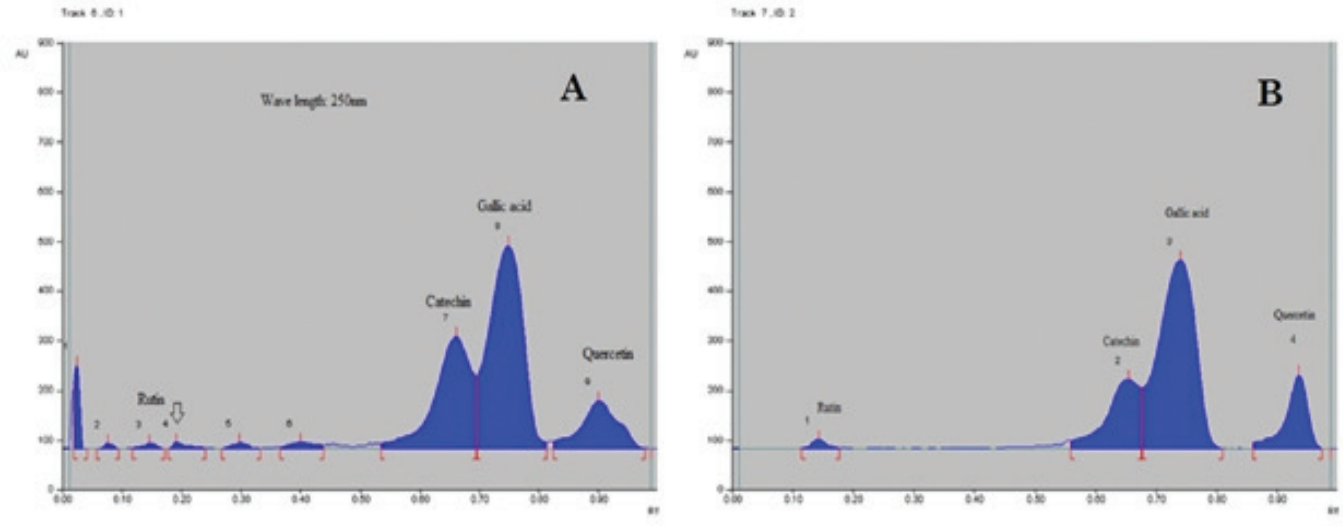

Fig. 2. [(A) Destinogram of AEGF at 254 nm (B) Destinogram of MEGF at 254 nm].

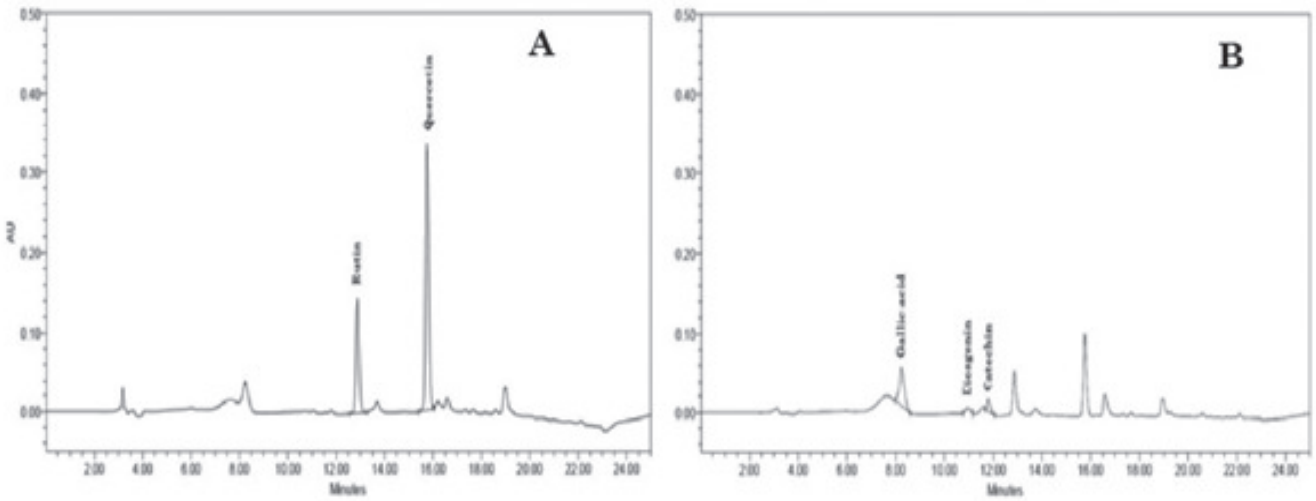

Fig. 3. HPLC chromatogram of standard marker compounds. [(A) at 254nm (Rutin and quercetin) and (B) at 278nm for gallic acid, catechin and diosgenin].

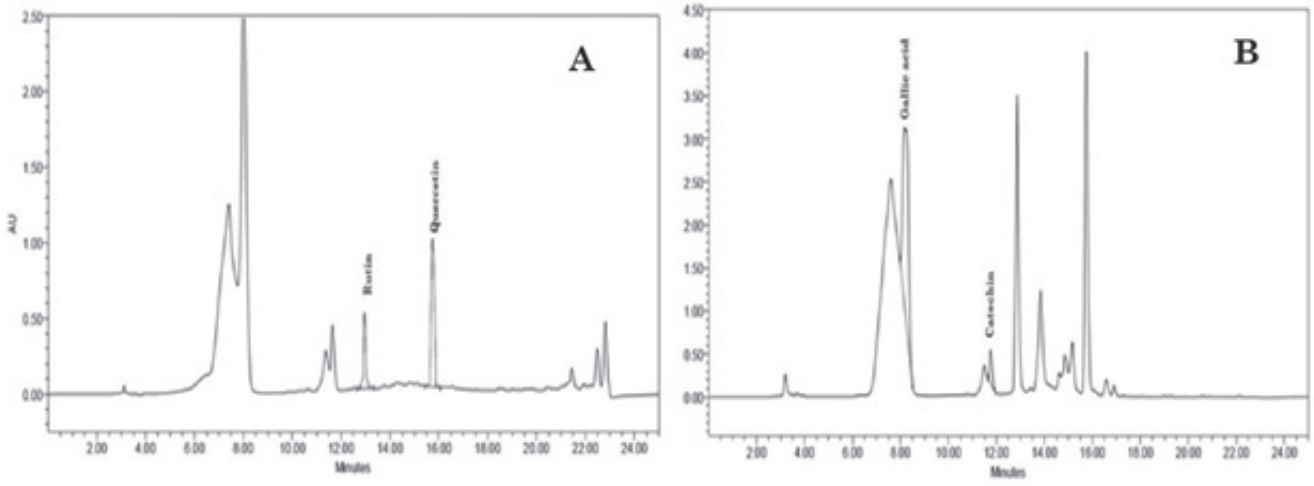

Fig. 4. HPLC chromatogram of AEGF compounds. [(A) at 254nm (Rutin and quercetin) and (B) at 278nm for gallic acid, catechin and diosgenin].

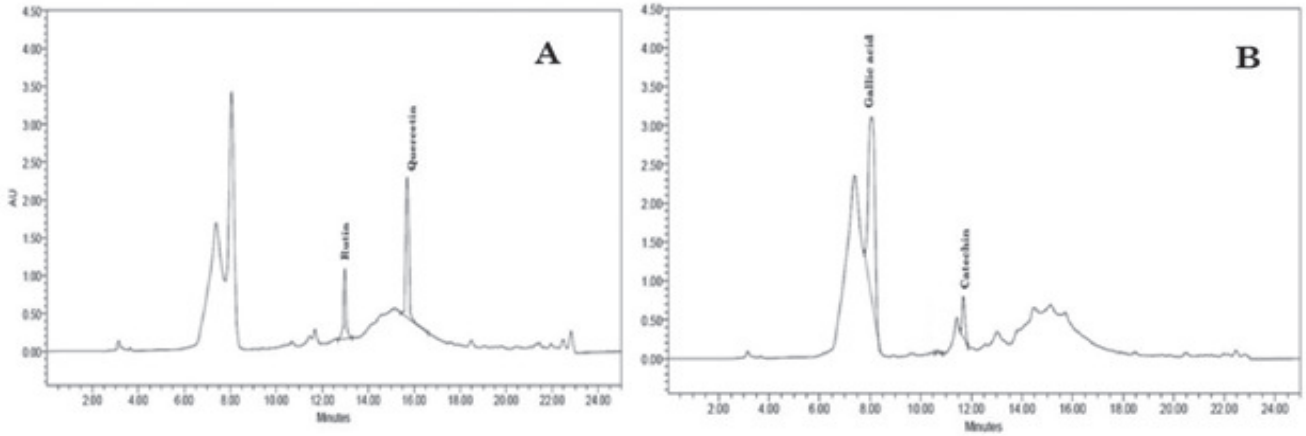

Fig. 5. HPLC chromatogram of MEGF compounds. [(A) at 254nm (Rutin and quercetin) and (B) at 278nm for gallic acid, catechin and diosgenin].

hypercalciuria and hyperoxaluria, which subsequently increased the excretion and retention of oxalate and promoted the nucleation and stone formation process [23]. The study also revealed that due to hyperoxaluria condition, increased uric acid and phosphate levels in urine, which further promotes calcium phosphate formation which leads to stone deposition in the kidney, uric acid levels interfere with calcium oxalate solubility in urine [23, 24]. Our results data (Table 12) showed, that in the disease control group oxalate, calcium, Phosphate, and uric acid levels significantly ($p< 0.001$) increased which promoted stone formation, while treatment with AEGF and MEGF significantly ($p< 0.001$) reduced the levels.

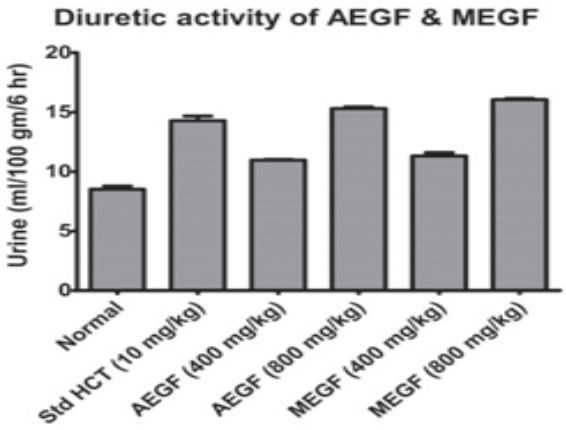

Fig. 6. Diuretic effect of AEGF, MEGF and standard (cystone) in rats.

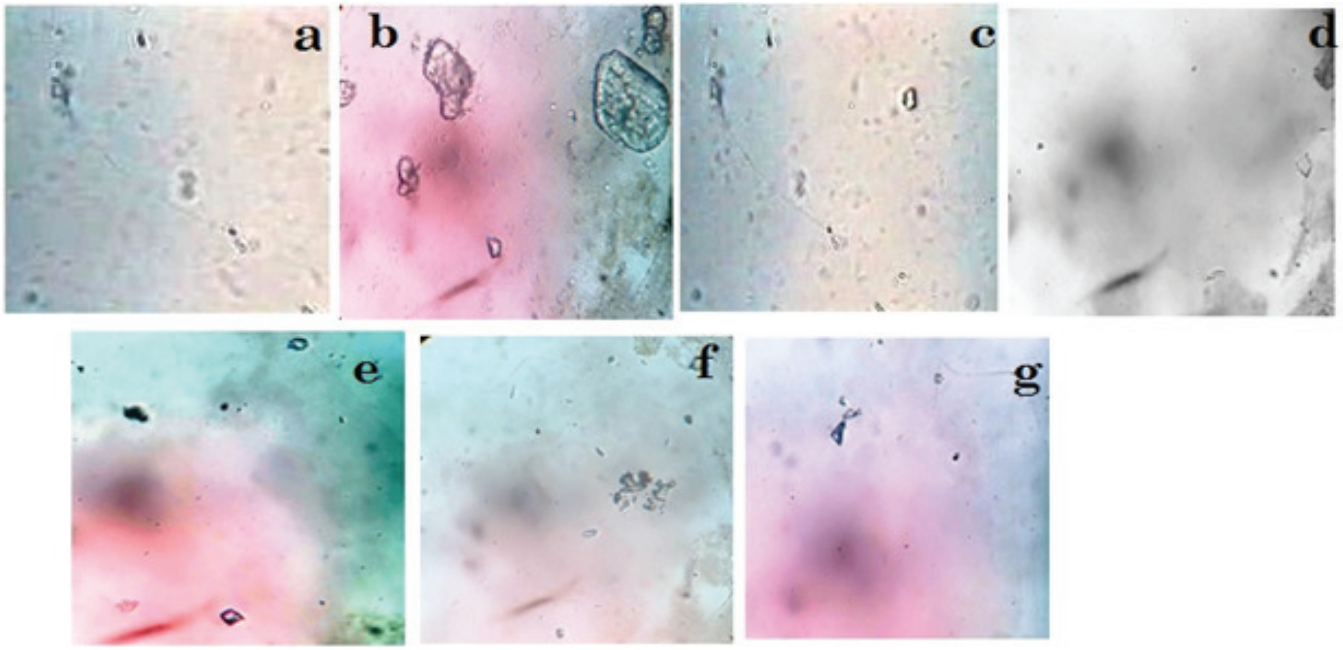

Fig. 7. Calcium oxalate crystal observed under microscope in 24 hr urine of rat. [(a) Normal control group showed absence of crystal (b) Disease control group showed large crystal and (c) Standard group (cystone 750 mg/kg) (d) AEGF (400 mg/kg) (e) AEGF (800 mg/kg) (f) MEGF (400 mg/kg) & (g) MEGF (800 mg/kg) showed less number of crystals in urine].

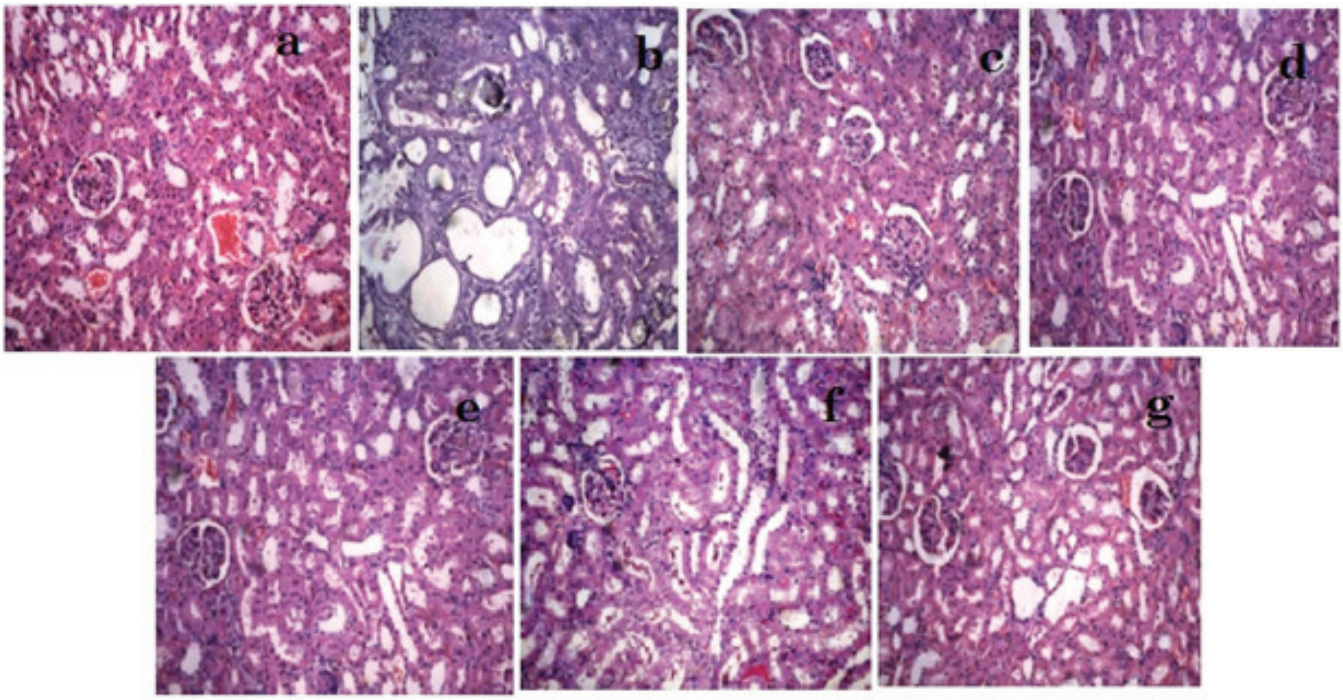

Fig. 8. Histology of rat kidney. [(a) Normal group (b) Disease control group showed crystal deposition having large size (c) standard group (cystone 750 mg/kg) (d) AEGF (400 mg/kg) (e) AEGF (800 mg/kg) (f) MEGF (400 mg/kg) & (h) MEGF (800 mg/kg) showed less number of crystal and normal structure of kidney].

Magnesium combines with oxalate to generate the soluble complexes that prevent super-saturation and decrease nucleation, while citrate makes soluble complexes with calcium and increases the urinary pH. [24, 25, 26] Administration of sodium oxalate caused hypo-magnesuria, and hypo-citraturia which led the stone formation in the disease control group, while treatment with AEGF, MEGF, and cystone showed significantly ($p<0.001$) increased the levels of magnesium and citrate (Table 12).

Patients with renal stones had decreased urine production as a result of decreased glomerular filtration rate, which led to an accumulation of waste products in the blood, including urea and creatinine, and a decrease in creatinine clearance [27]. In comparison to the disease control group, the administration of AEGF and MEGF significantly reduced the serum urea and increased creatinine clearance. The diuresis effect of AEGF and MEGF may be responsible for this result (Table 12).

Effect on kidney homogenate parameter

The kidney tissue of the disease control group had significantly ($p<0.001$) higher levels of urolithiasis promoters, such as oxalate, calcium, phosphate, and uric acid than the normal group. In the renal tissue of treatment groups, however, such promoters were shown to be considerably ($p<0.001$) lowered in a dose-dependent way as compared to the disease control group. The antioxidant enzyme catalase's activity was dramatically reduced ($p<0.001$) in the disease control group after receiving sodium oxalate [28], but it increased significantly in the treatment groups, protecting the tissue from oxidative alterations. (Table 12).

Effect on histopathology of kidney

The kidney's section study in the normal group revealed the kidney's architecture to be normal (Fig. 8a), but the administration of a sodium oxalate agent in the disease control group severely damaged the kidney's medulla, glomeruli, tubules, and interstitial spaces. In the kidney section of the disease control group, there was intratubular crystal deposition as well (Fig. 8b), whereas in the sections of the AEGF and MEGF treated groups, there was significant damage restored and less intratubular crystal deposition. [Fig. 8(d,e,f,g)] renal injury was discovered to have recovered in the standard group part, where crystal deposition was not observed (Fig. 8c).

Various studies reveal that flavonoids like rutin, quercetin, hyperoside, and diosmin have strong anti-oxidant and antilithiatic properties [19, 29] and saponins are known for breaking down mucoproteins, which are

essential parts of the stone matrix and have antilithic qualities. Other studies reported that Adenosine A1 receptor (AA1R) antagonists cause diuretic effects and protect the kidney. Certain flavonoids and their derivatives can compete with one another for the binding of the AA1R receptor and act as a potent antagonist and given diuretics activity [30]. Phyto-constituents test, HPTLC, and HPLC data reveal that both extracts contain flavonoids and saponins. The free radical scavenging, anti-inflammatory, anti-microbial activity, diuretics, and AA1R antagonist activity of flavonoids play remarkable roles in the prevention of crystal formation and dissolution of crystals.

CONCLUSION

Based on the above results and discussion, it can be concluded that AEGF and MEGF have anti-urolithiasis activity in NaOX-induced urolithiasis by promoting various urolithiasis inhibitors like magnesium and citrate and suppressing various urolithiasis promoters like calcium, oxalate, phosphate in 24 hr urine, serum, and renal tissue. In addition, the diuretic activity of AEGF and MEGF helps to flush out stone promoters in urine and decrease the super-saturation level of ions in urine and this prevents new CaOX nuclei formation. The diuretic activity also smooths the surface of CaOX due to that decrease in renal tissue damage and also increases the CaOX stone dissolution. HPTLC and HPLC analysis of extracts revealed the presence of flavonoids which complies with the protective effects observed in the *in vitro* and *in vivo* studies of extracts. Thus, the current result highlights that the seeds of *Grewia flavescens* have potential and beneficial effects in the prevention of CaOX stone formation in the kidney. However, further chemical analysis and biomarker-based assays are needed to establish the exact role of such bioactive in the prevention of renal stones.

ACKNOWLEDGMENT

The authors thank the Institute of Pharmacy, Nirma University for providing the infrastructure and technical facilities to accomplish the research. This study is a part of the research work of Dr. Vaibhavkumar B. Patel carried out at the Institute of Pharmacy, Nirma University, and to be submitted to the Institute as a part of the Ph.D. work.

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Cite this article as: Patel VB, Acharya N. Anti-urolithiasis and diuretic activities of *Grewia flavescens* roots. Explor Anim Med Res. 2024; 14(1), DOI: 10.52635/eamr/14.1.124-136.