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ORIGINAL ARTICLE

Antiurolithic Evaluation of *Cucurbita pepo* Seeds Extract against Sodium Oxalate-Induced Renal Calculi

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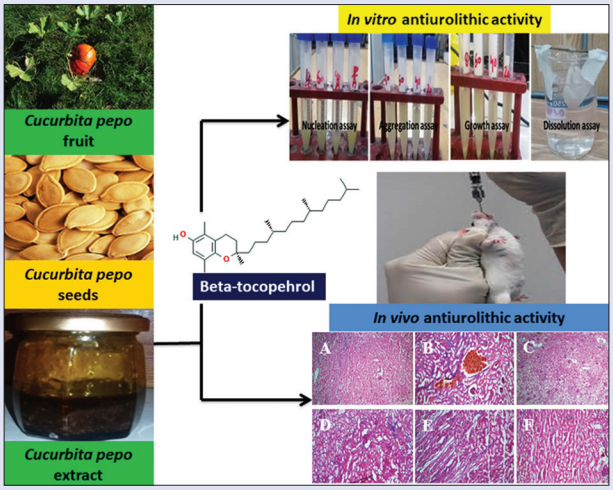
ABSTRACT

Background: Renal calculi, a painful kidney stone disease has worldwide health hazard. *Cucurbita pepo* is used for the management of lower urinary tract disease, benign prostatic hyperplasia, and micturition difficulty. **Objective:** The present study was investigated to resolve the antiurolithic potential of methanol extract of *C. pepo* (MECP) seed against sodium oxalate-induced renal calculi using both *in vitro* and *in vivo* models. **Materials and Methods:** MECP was prepared by macerating *C. pepo* seed powder in methanol. Gas chromatography coupled with mass spectrometry (GC-MS) analysis was performed to characterize the phytochemical profile of MECP. *In vitro* techniques such as nucleation, aggregation, growth, and dissolution assays were performed using different concentrations (20, 40, 60, and 80 mg/mL) of MECP and the standard drug cystone to determine their calcium oxalate (CaOx) crystals inhibitory potential. In male albino rats, calculi were induced by intraperitoneal administration of sodium oxalate (70 mg/kg) for 10 days. Various doses (250, 500, and 1000 mg/kg) of MECP were administered orally to male albino rats. Various pathological parameters such as body and kidney weights, serum (creatinine, blood urea nitrogen, and uric acid), urinary (calcium, potassium, oxalate, sodium, magnesium, phosphate, pH, and volume) analysis, and kidney histopathology were executed. **Results:** GC-MS fingerprints showed that beta-tocopherol, stigmasterol, and squalene are the major phytochemicals found in MECP. Results demonstrated that MECP significantly inhibited various steps of CaOx crystal formation such as nucleation, aggregation, growth, and dissolution in dose-dependent manner. MECP normalized the raised levels of oxalate, calcium, sodium, phosphate, uric acid, restored alterations in histopathology while elevated the reduced levels of magnesium, urine volume, and pH. **Conclusion:** The undertaken study rationalized the usage of *C. pepo* as an alternative or adjuvant treatment for renal calculi after clinical trials in human subjects.

Key words: Antiurolithic, *Cucurbita pepo*, methanol extract, renal calculi, sodium oxalate

SUMMARY

- *Cucurbita pepo* seed extract exhibited remarkable antiurolithic activity against sodium oxalate-induced renal calculi
- The antiurolithic activity of *Cucurbita pepo* seed extract may be due to its major phytochemicals beta-tocopherol, stigmasterol, and squalene.



Abbreviations used: MECP: Methanol extract of *Cucurbita pepo*; CaOx: Calcium oxalate; GC-MS: Gas chromatography coupled with mass spectrometry; NaOx: Sodium oxalate; STD: Standard; BUN: Blood urea nitrogen; S. creatinine: Serum creatinine; Ca^{+2}: Calcium; K^{+}: Potassium; Ox: Oxalate; Na^{+}: Sodium; Mg^{+2}: Magnesium; PO_4^{-2}: Phosphate.

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INTRODUCTION

The presence of calculi in any part of the urinary system for instance located in the ureter, kidney, and bladder referred to as ureterolithiasis, nephrolithiasis, and cystolithiasis, respectively. Mainly 75%–90% stones are formed of calcium oxalate (CaOx), 5%–20% of uric acid, 1% of apatite (calcium phosphate), 2% of struvite (magnesium ammonium phosphate) as well as 0.5%–1% of cystine.[1] Globally, it is the third devastating ailment affecting the population in Asia (1%–5%), Europe (5%–9%), Canada (12%), and Saudi Arabia (20%).[2] Epidemiologically, males (12%) are at greater risk as compared to females (6%). This disease most frequently affects 20–40 years of age. Multiple factors, including environment, specific genes, infections, concomitant diseases, nutrition, socio-economic, and certain medications, are responsible for renal calculi.[3]

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Its mechanism is attributed to many physiological phenomena such as supersaturation of urine with calculi-producing minerals, nucleation, aggregation as well as the growth of CaOx crystals which are inhibited in normal individuals due to the presence of inhibitors in urine.[4] This disease occurs mainly due to an imbalance between natural inhibitors such as magnesium, citrate, osteopontin, pyrophosphate, acid polypeptides, glycosaminoglycans, less pH, and volume of urine as well as promoters such as calcium, oxalate, phosphate, potassium, sodium, and uric acid.[5] Clinically, lithotripsy and surgical removal of renal calculi are mainstay therapies, but its rate of recurrence (10%) is a major hindrance.[6] Although medications such as thiazide diuretics, cystone, citrate, and allopurinol are effective therapeutic agents, they are not beyond side effects and consequently cannot be used for a longer period.[7]

Natural products are a good source of novel therapeutic agents. According to the WHO, plants have been used by 70%–95% of population in developing countries for curing numerous diseases from ancient times because of their extended activities, cost-effectiveness, and less side effects as compared to medicines prepared commercially.[8] Numerous plant extracts and phytochemicals have been reported for their antiurolithiatic potential.[9-12] *Cucurbita pepo* L. commonly referred to as pumpkin, summer squash, and vegetable marrow of Cucurbitaceae family, have chemical constituents such as fatty acids, carotenoids, tocopherols as well as phytosterols. It is used in urinary tract diseases such as benign prostatic hyperplasia,[13-15] diuresis,[16] and for nephroprotection.[17,18] *C. pepo* has beneficial activities such as anti-inflammatory, anti-diabetic, anti-bacterial, anti-ulcer, antioxidant, anti-tumor as well as anti-hyperlipidemic[19,20] attributable to the presence of various chemical constituents such as terpenoids, cucurbitacin glycosides, flavonoids, polyphenols, and cardiac glycosides. *C. pepo* seeds contain omega-6 and omega-3 fatty acids, L-tryptophan, and tocopherol or Vitamin E.[21] Vitamin E inhibits the retention of CaOx in renal tissues through acting as antioxidant and removing peroxidative membrane injury along with maintaining the level of glutathione peroxidase, thus preventing kidney stone formation.[22] Due to the presence of flavonoids, polyphenols and Vitamin E which have a role in dissolving kidney stones, the present study has been designed to evaluate the antiurolithic potential of *C. pepo* seed against sodium oxalate-induced renal calculi.

MATERIALS AND METHODS

Collection of plant material and preparation of extract

C. pepo was collected from Ayub Agriculture Research Institute, Faisalabad, Pakistan, in August and identified by Prof. Dr. Mansoor at the Department of Botany, University of Agriculture Faisalabad, Pakistan. Voucher specimen (No. 520-1-2018) was kept at the Department of Botany, University of Agriculture Faisalabad, Pakistan. From *C. pepo* fruits, seeds were washed well with distilled water, dried under shade, and then powdered. Powder (500 g) was macerated with methanol (2500 mL) for 4–5 days. Macerate was filtered through Whatman filter paper No. 1 and filtrate was concentrated using rotary evaporator at 40°C to obtain methanol extract of *C. pepo* (MECP).

Quantitative phytochemical analysis

MECP was evaluated for estimating total flavonoids,[23] total phenols,[24] total alkaloids, and total glyco saponins.[25]

Gas chromatography coupled with mass spectrometry analysis

Total chemical constituents along with their molecular weight and molecular weight were determined by Gas chromatography coupled with mass spectrometry (GC-MS). The temperature was maintained at 70°C–280°C with helium taken as the carrier gas. Compounds in MECP were identified by comparing their mass spectra and retention times with known compounds as well as throughcomputerized matching through NIST libraries.

In vitro evaluation of methanol extract of *Cucurbita pepo* against calcium oxalate crystals

Nucleation or turbidity assay

Nucleation assay was performed through the method described by Patel *et al.*[26] Solutions of each of 7.5 mmol/L of sodium oxalate (NaC_2O_4) as well as 5 mmol/L calcium chloride ($CaCl_2$) were prepared within buffer consisting of Tris-HCl (0.05 mol/L) along with NaCl (0.15 mmol/L) having pH 6.5. Various concentrations such as 20, 40, 60, and 80 mg/mL of MECP and cystone tablets prepared in distilled water used as standard were mixed with 3 mL of $CaCl_2$ solution. Then, 3 mL of NaC_2O_4 solution was added for initiating crystallization. Finally, mixture was incubated at 37°C for 30 min. The optical density (OD) of MECP was compared with OD of standard and was determined at 620 nm. The percentage inhibition of nucleation of CaOx crystals was anticipated through the following formula:

$$\%\text{ age inhibition} = 1 - \frac{\text{OD (MECP)}}{\text{OD (Cystone)}} \times 100$$

Aggregation assay

Aggregation assay was performed via the method described by Saha and Verma[27] Solutions of each of 50 mmol/L of $CaCl_2$ as well as NaC_2O_4 were added together, equilibrated in water bath at 60°C for one h, incubated at 37°C all over the night followed by evaporated to yield CaOx crystals. Then, 1 mg of CaOX crystals was mixed with 1 mL of buffer consisting of Tris HCl 0.05 mol/L and NaCl 0.15 mol/L having pH 6.5. Variant concentrations such as 20, 40, 60, and 80 mg/mL of MECP and standard were mixed with 3 mL of CaOx solution and then incubated at 37°C for 30 min. The OD of MECP compared with OD of the standard was measured at 620 nm. The percentage inhibition of aggregation of CaOx crystals was anticipated through formula represented in nucleation assay.

Growth assay

The growth assay was performed via the method described by Chaudhary *et al.*[28] Solutions of 1 mL of each of 4 mM of $CaCl_2$ as well as NaC_2O_4 were added to 1.5 mL of buffer containing NaCl (10 mM) and Tris-HCl (10 mM) having pH 7.2. In this 30 µL of CaOx, crystals were added. Variant concentrations such as 20, 40, 60, and 80 mg/mL of MECP and standard were mixed with mixture having CaOx crystals and then incubated at 37°C for 30 min. The OD of MECP compared with OD of the standard was measured at 240 nm. The percentage inhibition of aggregation of CaOx crystals was anticipated via formula represented in nucleation assay.

Dissolution assay

Dissolution assay was performed via the method with some modifications described by Phatak and Hendre.[29] First, semipermeable membranes of eggs were prepared. The solution of CaOx was prepared by homogenous precipitation. A total of 1.47 g of $CaCl_2$ was mixed with 100 mL distilled water, as well as 1.42 g of disodium hydrogen phosphate, was mixed with 100 mL of 2 N H_2SO_4. Both solutions were combined for precipitation of CaOx which was rinsed with ammonia solution for removing acid contents and then with distilled water, subsequently dried at 60°C for 2 h. Test tubes were divided into five groups:

- Group I served as control and 1 mg/mL of CaOx + 1 mL of distilled water
- Group II served as standard and 1 mg/mL of CaOx + 1 mL of cystone
- Group III-V served as tested and 1 mg/mL of CaOx + 1 mL of MECP (20, 40, 60, and 80 mg/mL).

The entire contents of three test tubes were placed separately in egg membrane which was knotted via thread, kept in hold via stick in beaker having 0.1 M Tris-HCl buffer (150 mL). The beakers were placed in an incubator for 3 days. The contents from membranes were shifted into test tubes separately. Then, 2 mL of 1 N H_2SO_4 to each test tube and titrated with 0.9494 N $KMnO_4$ till a light pink color endpoint was obtained. The amount of remaining undissolved CaOx is subtracted from the total quantity used in the experiment to determine the total quantity of dissolved CaOx via MECP. Each mL of 0.9494 N $KMnO_4$ is equal to 0.1898 mg of CaOx.

In vivo evaluation of methanol extract of *Cucurbita pepo* against calcium oxalate crystals

Experimental animals

Healthy male albino rats of 150–200 g were purchased from animal house of University of Agriculture Faisalabad. The study was conducted after getting approval from the Animal Ethics Committee of GCUF with reference number GCUF/ERC/1995. They were housed in individual cages for 5 days in standard conditions of room having temperature 22°C ± 4°C, relative humidity 30%–70%, and 12 h light and dark cycle. They were fed laboratory food with an unrestricted supply of drinking water.

Study design

Rats were placed individually in metabolic cages. During the experiment, food and water were given freely in cages and body weights, water, and diet intake was determined. After 1 week of acclimatization, sodium oxalate (70 mg/kg) was administered intraperitoneally for 10 days to induce renal calculi. The rats were divided into six groups ($n = 6$).

- Group I: Normal control
- Group II: Disease control
- Group III: Rats having renal calculi and administered with cystone (700 mg/kg)
- Group IV: Rats having renal calculi and administered with MECP (250 mg/Kg)
- Group V: Rats having renal calculi and administered with MECP (500 mg/Kg)
- Group VI: Rats having renal calculi and administered with MECP (1000 mg/kg).

Determination of body and kidney weights

Before, during and after the completion of experiment of 10 days, body weights of rats were measured. After that, all the rats were scarified by cardiac puncture. Weights of the right and left kidneys of all the rats were also measured.

Determination of serum creatinine, blood urea nitrogen, uric acid, and total proteins

The blood samples of rats were taken for determining levels of serum creatinine (S. creatinine), blood urea nitrogen (BUN), uric acid, and total proteins.

Determination of urinary calcium, potassium, oxalate, sodium, magnesium, phosphate, pH, and volume

One day before the completion of the experiment of 10 days, urine was collected after 24 h. pH of urine using pH meter and total volume using measuring cylinder was determined. Urine parameters such as levels of Ca^{+2}, K^{+}, Ox, Na^{+}, Mg^{+2}, and PO_4^{-2} were calculated. Furthermore, complete urine examination was carried out for the screening of the presence of ketones, albumin, crystals, pus cells, and urobilin in urine.

Histopathological analysis

Right kidneys isolated from sacrificed rats were fixed in 10% formalin then after processing embedded in paraffin wax. Paraffin sections were made at 5 mm and stained with hematoxylin and eosin. The slides were studied under a light microscope and captured the magnified images of tissues.

Statistical analysis

All the results were represented as mean ± standard error of the mean. The statistical significance between groups was evaluated using two-way analysis of variance using Bonferroni posttests via Graphpad prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

Quantitative phytochemical analysis

The percentage yield of MECP was 18%. Quantitative phytochemical analysis of MECP exhibited that total phenols, total flavonoids, and total alkaloids were determined using linear regression equation which was obtained from gallic acid, quercetin, and atropine standard curves, respectively. Concentrations of total phenols, total flavonoids, total alkaloids, and total glycosaponins were 86 mg GAE/g (gallic acid equivalent per g), 36.10 mg QE/g (quercetin equivalent per g), 96.44 mg AE/g (atropine equivalent per g), and 0.362 g, respectively [Table 1].

Gas chromatography coupled with mass spectrometry fingerprints

GC-MS analysis chromatogram shown in Figure 1 revealed that eight major chemical compounds were identified in MECP. Their molecular weights, molecular formulas, retention times along with their percentages are illustrated in Table 2.

In vitro evaluation of methanol extract of *Cucurbita pepo* against calcium oxalate crystals

Results of the nucleation assay showed that percentage inhibition of CaOx crystals by MECP was greater (94% ± 2.03%) significantly $^{***}P < 0.001$ at concentration of 80 mg/mL as compared to the standard drug (96% ± 1.53%). Outcomes of aggregation assay revealed that percentage inhibition by MECP was more (87% ± 1.76%) significantly $^{***}P < 0.001$ at concentration of 80 mg/mL as compared to standard (96% ± 0.88%). Results of growth assay exhibited that percentage inhibition by MECP was more (93% ± 1.45%) significantly $^{**}P < 0.01$ at concentration of 80 mg/mL as compared to standard (98% ± 0.58%). Results of nucleation, aggregation, and growth assay are described in Table 3 and shown in Figure 2. Outcomes of dissolution assay exhibited that percentage inhibition of MECP was more (0.31% ± 0.08%) at high concentration, i.e., 80 mg/mL than standard drug (0.24% ± 0.07%), described in Table 4 and shown in Figure 3.

Table 1: Quantitative phytochemical analysis of methanol extract of *Cucurbita pepo* seed

Phytochemical	Concentration	Regression coefficient (R^2) value
Total flavonoids	36.10 (mg QE/g)	0.976
Total phenols	86. 00 (mg GAE/g)	0.971
Total alkaloids	96.44 (mg AE/g)	0.975
Total glycosaponins	0.362 (g)	-

QE: Quercetin equivalent; GAE: Gallic acid equivalent; AE: Atropine equivalent

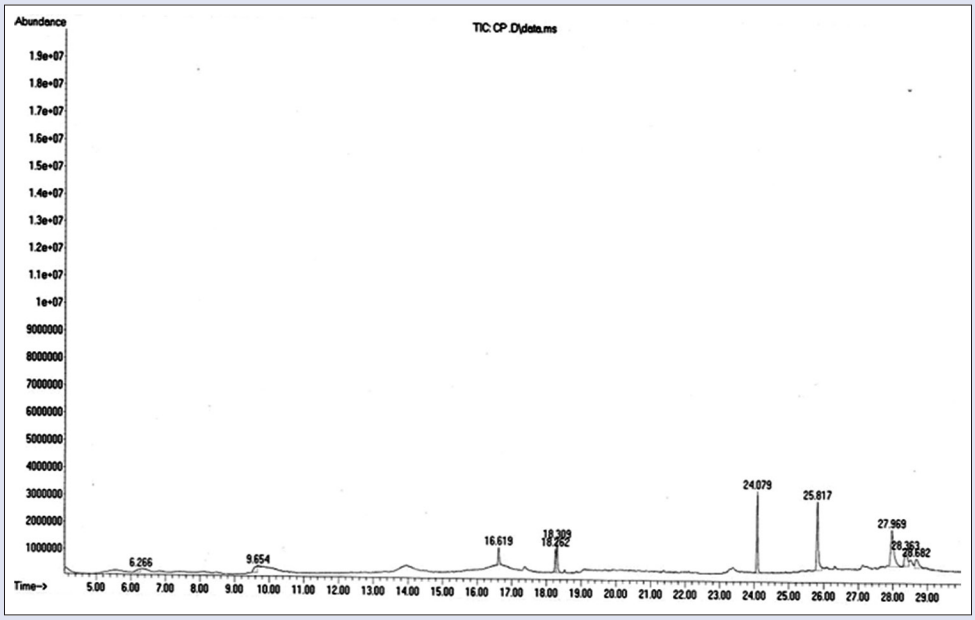

Figure 1: Gas chromatography coupled with mass spectrometry chromatogram of methanol extract of *Cucurbita pepo* seed

Table 2: Gas chromatography coupled with mass spectrometry analysis of methanol extract of *Cucurbita pepo* seed

Peak number	Retention time (min)	Compound	Molecular weight	Molecular formula	Percentage
1	6.266	Propylene carbonate	102	$C_4H_6O_3$	2.307
2	9.654	5-Hydroxymethyl-2-furaldehyde	126	$C_4H_6O_3$	4.077
3	16.619	Pentadecanoic acid, 14-methyl-, methyl ester (Palmitic acid methyl ester)	270	$C_{17}H_{34}O_2$	4.137
4	18.262	9, 12-octadecadienoic acid, methyl ester	294	$C_{19}H_{34}O_2$	5.191
5	18.309	10-Octadecenoic acid, methyl ester	296	$C_{19}H_{34}O_2$	7.124
6	24.079	Squalene	410	$C_{30}H_{50}$	16.915
7	25.817	Beta-tocopherol	416	$C_{28}H_{48}O_2$	22.589
8	27.969	Stigmasterol	412	$C_{29}H_{48}O$	22.796

GC-MS: Gas chromatography coupled with mass spectrometry

Table 3: Effect of methanol extract of *Cucurbita pepo* seed on the inhibition of nucleation, aggregation, and growth calcium oxalate crystals

Concentration (mg/mL)	Nucleation assay (%)		Aggregation assay (%)		Growth assay (%)	
	MECP	STD	MECP	STD	MECP	STD
20	65±1.73**	84±2.08	63±2.40***	76±2.60	75±1.76**	83±1.76
40	74±2.33*	82±2.03	75±1.76	82±1.45	85±1.73**	93±1.76
60	85±2.65***	87±1.76	82±0.88**	91±2.08	91±0.58*	97±0.88
80	94±2.03**	96±1.53	87±1.76**	96±0.88	93±1.45	98±0.58

Values are expressed as mean±SEM (n=3) ***P<0.001, **P<0.01, *P<0.05 when compared with standard. STD: Standard; MECP: Methanol extract of *Cucurbita pepo* seed; SEM: Standard error of the mean

Table 4: Effect of methanol extract of *Cucurbita pepo* seed on the dissolution of calcium oxalate crystals

Groups	Amount of CaOx (mg) dissolved	Dissolution of CaOx (%)
Control	0	0
STD	0.76	76
MECP (20 mg/mL)	0.45	45
MECP (40 mg/mL)	0.49	49
MECP (60 mg/mL)	0.54	54
MECP (80 mg/mL)	0.58	58

Values are expressed as mean±SEM (n=3) ***P<0.001, **P<0.01, *P<0.05 when compared with standard. CaOx: Calcium oxalate; STD: Standard; MECP: Methanol extract of *Cucurbita pepo* seed; SEM: Standard error of the mean

In vivo evaluation of methanol extract of *Cucurbita pepo* against calcium oxalate crystals

The bodyweight of disease group was decreased significantly **P < 0.001 during the study than the normal control group. However, body weights of standard and MECP-treated groups remain constant than the disease group. Weight of kidneys in disease group was also decreased significantly ***P < 0.001 than normal control group [Table 5]. In disease group, a significant increase ***P < 0.001 in the levels of BUN, uric acid, and S. creatinine (25.14 ± 1.01, 1.62 ± 0.04, and 1.23 ± 0.02 mg/dL) was observed than normal control group (10.44 ± 1.03, 0.84 ± 0.11, and 0.75 ± 0.05 mg/dL). In disease group, total proteins (7.91 ± 0.09 mg/dL respectively) increased non-

Figure 2: Nucleation, aggregation, growth, and dissolution assay of *Cucurbita pepo* seed

significantly than normal control group (6.12 ± 0.12 mg/dL). While in standard drug and MECP treated groups, levels of S. creatinine, uric acid, and total proteins were decreased significantly than disease group [Table 6].

In disease group, a significant increase ***$P < 0.001$ in the levels of Ca^{+2}, K^{+}, Ox, Na^{+} and PO_4^{-2} in urine (14.49 ± 0.88 mg/dL, 88.00 ± 1.24 mmol/L, 2.14 ± 0.05 mg/dL, 176.33 ± 2.72 mmol/L, and 1.65 ± 0.04 mmol/L, respectively) were observed than normal control group (6.80 ± 0.54 mg/dL, 42.33 ± 1.48 mmol/L, 1.31 ± 0.06 mg/dL, 119.83 ± 3.46 mmol/L, and 0.72 ± 0.06 mmol/L, respectively). In disease group, a significant decrease in ***$P < 0.001$ the level of Mg^{+2} (63.33 ± 1.56 mmol/L) than the normal control group (89 ± 2.34 mmol/L). While in standard drug and MECP

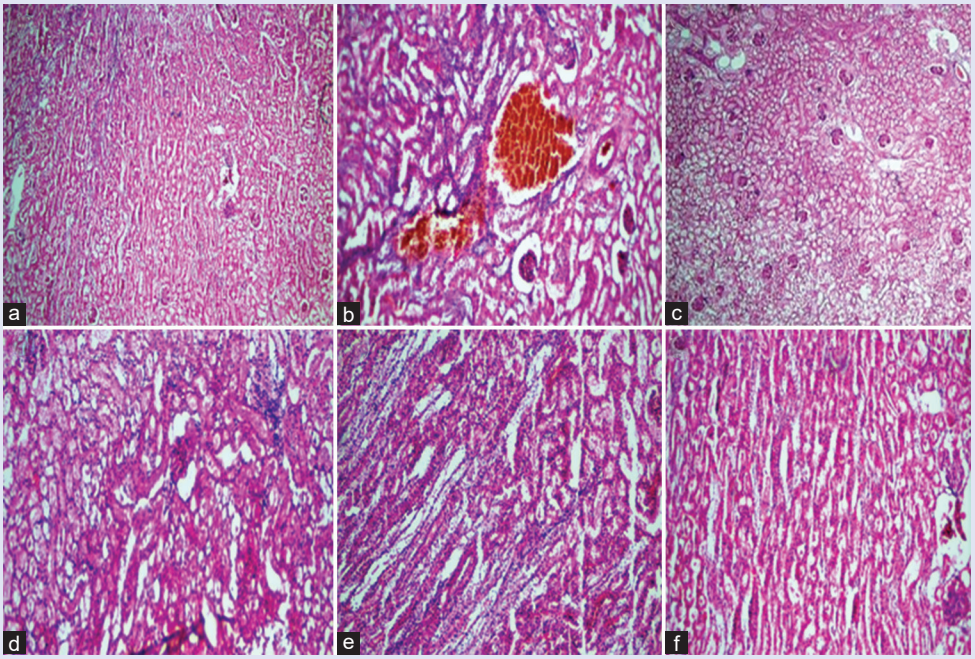

Figure 3: Histopathological examinations of kidney tissues. (a) Normal control, (b) Disease control, (c) Cytone (700 mg/kg) treated, (d) methanol extract of *Cucurbita pepo* (250 mg/kg) treated, (e) methanol extract of *Cucurbita pepo* (500 mg/kg), (f) methanol extract of *Cucurbita pepo* (1000 mg/kg) treated

Table 5: Effect of methanol extract of *Cucurbita pepo* seed on body weights of rats

Groups	1st day body weight (g)	5th day body weight (g)	10th day body weight (g)	Kidney weights (g)	
				Right	Left
Normal control	152±1.06	149±1.05	150±1.06	0.53±0.01	0.46±0.01
Disease control	165±2.94	157±3.53***	146±2.51*	0.53±0.01	0.48±0.01
STD	160±1.18	153±1.08	154±1.57**	0.63±0.01***	0.56±0.01***
MECP (250 mg/kg)	158±1.58	152±1.67	155±1.65	0.63±0.01***	0.58±0.01***
MECP (500 mg/kg)	151±1.82***	148±1.17**	155±1.70**	0.68±0.01***	0.58±0.02***
MECP (1000 mg/kg)	164±1.82*	160±2.32	152±1.63**	0.57±0.01**	0.48±0.02

Values are expressed as mean±SEM (n=6) ***P<0.001, **P<0.01, *P<0.05 when compared with disease control group. STD: Standard; MECP: Methanol extract of *Cucurbita pepo* seed; SEM: Standard error of the mean

Table 6: Effect of methanol extract of *Cucurbita pepo* seed on blood urea nitrogen, serum creatinine, uric acid, and total proteins

Parameters (mg/dL)	Normal control	Disease control	STD	MECP		
				250 (mg/kg)	500 (mg/kg)	1000 (mg/kg?)
S. creatinine	0.75±0.05	1.28±0.02	0.69±0.04***	0.68±0.02***	0.77±0.06***	0.84±0.07***
BUN	10.44±1.03	25.14±1.01	11.89±1.06***	10.77±0.69***	13.98±1.47***	15.68±0.82***
Uric acid	0.84±0.11	1.62±0.04	0.81±0.11***	0.92±0.07***	1.09±0.07***	1.21±0.07***
Total proteins	6.12±0.12	7.91±0.09	6.41±0.06	6.58±0.04	7.07±0.01	7.31±0.07

Values are expressed as mean±SEM (n=6) ***P<0.001, when compared with disease control group. STD: Standard; MECP: Methanol extract of *Cucurbita pepo* seed; BUN: Blood urea nitrogen; S. creatinine: Serum creatinine; SEM: Standard error of the mean

treated groups, levels of Ca^{+2}, K^{+}, Ox, Na^{+}, and PO_4^{-2} were decreased ignificantly as compared to disease group. In disease group, urine pH was 8.47 ± 0.11 which was close to the pH of normal control group while volume of urine was 7.77 ± 0.36 mL which was decreased than normal control group [Table 7].

Histopathological examination revealed that the normal control group showed no changes in renal tubules and glomerulus. There was no deposition of CaOx crystals in renal tissues [Figure 3a]. The disease group showed marked deposition of CaOx crystals, significant tubular dilation, congestion, and inflammation [Figure 3b]. The standard drug-treated group exhibited no hemorrhage and necrosis [Figure 3c]. There was no deposition of CaOx crystals. A total of 250 mg of MECP-treated group exhibited mild hemorrhage but no inflammation [Figure 3d]. A total of 500 mg of MECP-treated group also exhibited moderate hemorrhage but no inflammation [Figure 3e]. 1000 mg of MECP-treated group exhibited marked tubular necrosis and inflammation [Figure 3f]. Recovery of CaOx crystals in renal tissues was predominant at all three doses (250, 500, and 1000 mg) of MECP.

DISCUSSION

Standardization of plants is very essential so that consistent quality and effects can be achieved. Phytochemical analysis of MECP exhibited that it has remarkable amount of flavonoids, phenols, alkaloids, and glycosaponins, which have protective effect in preventing CaOx crystals formation in urolithiasis.[10] Flavonoids and phenols act as antioxidants through scavenging free radicals, enhancing endogenously produced antioxidant enzymes, chelating the metals, and decreasing membrane lipid oxidation. GC-MS analysis identified several compounds in MECP but majorly beta-tocopherol, a component of Vitamin E is present. Vitamin E act as an antioxidant *via* preventing peroxidative injury and re-establishing glutathione redox balance thus inhibited CaOx accumulation in renal tissues.[30,31] CaOx crystals formation is utmost prevailing type of all urinary stone diseases. The phenomena included in its pathological bio-mineralization are nucleation, aggregation as well as the growth of crystals.[32] The current study was conducted to report the phenomena taking place in CaOx stone development for investigating the efficacy of MECP as an antiurolithiatic agent. Nucleation is an initial step in the pathogenesis of CaOx crystals. Nucleation basically marks a thermodynamically driven event of phase change wherein dissolved substances in a supersaturated solution spontaneously crystallize.[33] Significant inhibition in the nucleation of CaOx crystals and calcium reduction was observed in the presence of MECP which was even better than in the presence of cystone. This suggests the anti-crystallization activity of MECP against CaOx crystallization. One possible mechanism of anti-crystallization activity of MECP could be its ability to form complex with free calcium and oxalate ions, thus preventing the formation of CaOx complexes, as has also been suggested for *Sarghassum wightti*.[34]

Aggregation of crystals marks the process wherein numerous crystals in the solution come together and adhere forming large crystal agglomerates. Aggregation is a key determinant of crystal retention as large crystal agglomerates are the ones that produce renal tubular obstruction, thereby promoting stone formation.[32] MECP showed a significant inhibitory effect on CaOx crystal aggregation. Growth of CaOx crystals marks the event of deposition of crystal-forming ions present in the supersaturated solution on preformed CaOx crystal lattice.[35] This event of CaOx crystals growth was also tracked in the present study. MECP exhibited growth inhibitory activity as it was also confirmed from the crystals of reduced size produced in the presence of MECP. Nucleation, aggregation, and growth of crystals were inhibited in a dose-dependent manner by MECP which corroborates to the results of the previous study.[36] Numerous methods have been used in rats to induce renal calculi. Among these methods, the administration of sodium oxalate intraperitoneally causes prompt development of CaOx crystals in tubules of the kidney of rats and therefore used for screening of anti-urolithiatic drugs.[37] The mechanism of sodium oxalate-induced renal calculi causes hyperoxaluria which is due to the poor solubility of oxalate in urine and its precipitation. Hyperoxaluria damage renal tubules and lead to nucleation, aggregation, and growth of CaOx crystals.[38] In the present study, level of oxalate in disease group is increased while in MECP-treated groups restored which are in agreement with previously published studies.[39] Increased urinary calcium is a factor favoring the nucleation and precipitation of calcium, oxalate, or phosphate from urine and consequent crystal growth. It has been reported that hyperabsorption of calcium is due to defective renal tubular reabsorption.[40] Hypomagnesaemia is a causative factor of renal calculi.[41] Decreased in magnesium level was detected in the disease group while MECP and standard groups improved magnesium levels in the present study which are close to prior studies.[7,39,42,43] Levels of uric acid, S. creatinine, BUN, and protein are increased in renal calculi owing to obstruction in tubules and reduced urine volume along with glomerular filtration rate. Histopathological studies exhibited that cystic dilation of renal tubules, inflammation, hemorrhage, and deposition of CaOx crystals were observed in the disease group which were ameliorated in standard drug and MECP-treated group in dose-dependent manner.[41,44]

CONCLUSION

Folklore use of *C. pepo* as diuretic, nephroprotective, and antiurolithic has been validated by its scientific investigation. *In vitro* study of MECP exhibited reduction in the nucleation, aggregation, growth, and dissolution of CaOx crytals. The extract significantly reduced the urolithiatic elements such as oxalate, sodium, calcium, phosphate, and uric acid. It is concluded that *C. pepo* has antiurolithic potential due to the presence of stone dissolving phytochemicals. The study suggests clinical trials of MECP which could be used as a potential candidate for antiurolithic drug development.

Table 7: Effects of methanol extract of *Cucurbita pepo* seed on urinary calcium, potassium, oxalate, sodium, magnesium, phosphate, pH, and volume

Parameters	Normal control	Disease control	STD	MECP		
				250 (mg/kg)	500 (mg/kg)	1000 (mg/kg)
Ca^{+2} (mg/dL)	6.80±0.54	14.49±0.88	9.87±0.33***	8.33±0.44***	10.61±0.29***	12.41±0.23***
K^{+} (mmol/L)	42.33±1.48	88±1.24	49.67±0.88***	51.17±2.39***	63.83±1.30***	65.67±1.45***
Ox (mg/dL)	1.31±0.06	2.14±0.05	1.27±0.06***	1.16±0.08***	1.45±0.04***	1.64±0.04***
Na^{+} (mmol/L)	119.83±3.46	176.33±2.72	136.33±3.57***	136.83±2.94***	146.50±2.47***	156.67±1.71***
Mg^{+2} (mmol/L)	86±2.34	63.33±1.56	98.83±2.86***	100±2.41***	106.67±3.61***	114.17±3.28***
PO_4^{-2} (g/L)	0.72±0.06	1.65±0.04	0.71±0.08***	1.02±0.07***	1.23±0.04***	1.44±0.03*
pH	7.48±0.16	8.47±0.11	8.01±0.11	7.31±0.16	8.12±0.03	7.93±0.06
Volume (mL)	10.08±1.15	7.77±0.36	11.55±0.11***	11.57±0.24***	12.48±0.19***	13.18±0.31***

Values are expressed as mean±SEM (n=6). ***P<0.001, *P<0.05 when compared with the disease control group. STD: Standard; MECP: Methanol extract of *Cucurbita pepo* seed; Ca^{+2}: Calcium; K^{+}: Potassium; Ox: Oxalate; Na^{+}: Sodium; Mg^{+2}: Magnesium; PO_4^{-2}: Phosphate; SEM: Standard error of the mean

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Conflicts of interest

There are no conflicts of interest.

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