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Antiurolithiatic activity of Indian medicinal plant: *Ocimum kilimandscharicum* Gurke (Lamiaceae)

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ABSTRACT

Urolithiasis is the most prevalent condition of the urinary system, characterized by the formation of stones inside the urinary tract. It is urgent to look for a natural urolithiasis therapy due to the serious side effects of conventional medications. Hydro-alcoholic (80% v/v) extract of the aerial parts of *Ocimum kilimandscharicum* (OK) and its ethyl acetate, chloroform, *n*-butanol, aqueous, and *n*-hexane fractions were subjected to in vitro antiurolithiatic screening as well as preliminary screening of phytochemicals. The in vitro antiurolithiatic activity of *O. kilimandscharicum* was studied using its hydroalcoholic extract (HAEOK). Calcium phosphate test using a colorimetric approach and calcium oxalate assay using a titrimetric model were used to determine the proportion of calcium oxalate crystals that dissolved. Total phenolic content (TPC) and total flavonoid content (TFC) were measured for the extract and fractions of OK. Ethyl acetate fraction (EAFOK) had a greater capacity to suppress crystal formation in both the calcium phosphate and calcium oxalate assays. The percent dissolution of calcium oxalate by HAEOK and EAFOK (31.48 ± 0.920% and 39.21 ± 0.903%) and calcium phosphate crystals by HAEOK and EAFOK (59.03 ± 0.820% and 66.62 ± 0.468%) was determined, respectively. At $p < 0.05$ and $p < 0.01$, differences between the results were regarded as significant. Cystone was employed as a standard drug. This study revealed that EAFOK showed significant antiurolithiatic activity. The antiurolithiatic activity of the extract/fraction was attributed to the steroids, triterpenoids, and flavonoid content of OK.

1. Introduction

Urolithiasis is an intricate mechanism that explains various physicochemical changes such as supersaturation, nucleation, aggregation, growth, and retention in kidneys (Butterweck & Khan, 2009). UL prevalence varies geographically, with 7%-13% in North America, 5%-9% in Europe, and 1%-5% in Asia. Africa's prevalence is unknown due to lack of medical facilities and research (Ondziel-Opara et al., 2022). Endogenous (calcium, phosphorous, and oxalate levels, elevated thyroid hormone levels, and difficulty in elimination of nitrogenous waste) and exogenous (excess water loss, Insufficient intake of fluid, extremely hot climate) factors are responsible for lithogenesis (Riaz et al., 2023).

Consumption of high oxalate-containing foodstuffs was also a major cause of the formation of uroliths (Aziz et al., 2024; Shabbir et al., 2023). The most common kind of urinary calculi illness is calcium oxalate (Iqbal et al., 2022). CaOx crystals adhere to renal tubular epithelial cells, forming a crucial stage in stone production. Randall's plaques (RPs) theory suggests these deposits enlarge and reach the renal papillary surface, attracting CaOx crystals, and forming CaOx stones. These theories are supported by research on reactive oxygen species and oxidative stress (Hong & Qin, 2023). The pathogenesis of urolithiasis includes the various steps involved in the emergence of stone including nucleation, growth, and aggregation of crystals

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(Kant et al., 2020; Shah et al., 2023). The nucleation phase is identified as a thermodynamically driven step in phase change wherein the dissolved supersaturated solution leads to crystallization (Bharathi et al., 2013; Rahim et al., 2023; Zahra et al., 2023). The production of urinary stones can be avoided by preventing supersaturation or subsequent phases in crystallization (Naveed et al., 2022a; Waseem et al., 2023; Zahid et al., 2022). Nowadays precautions have been taken to minimize the supersaturation of urine such as sufficient fluid intake and also by avoiding the consumption of dairy products (Smyslova et al., 2015).

As per the epidemiological studies report it affects around 12% of the population, with a reappearance rate of 70-81% in men, and 47-60% in women. Based on the chemical composition, urinary stones are further categorized into numerous types such as calcium oxalate (> 60%), calcium phosphate (10-20%), cysteine (1-5%), struvite (1-14%), uric acid (5-10%), and miscellaneous (4%) (Klein, 1996).

Advanced clinical research studies have demonstrated the therapeutic benefits of botanicals in preventing and managing of a wide range of diseases. More than 150 different species of *Ocimum* can be found in the genus, which ranges from sea level to a height of 6.000 feet and is found in temperate and subtropical areas of the world. *O. gratissimum*, *O. sanctum*, *O. basilicum*, *O. kilimandscharicum*, and *O. americanum* are examples of known important species of the genus *Ocimum* (Carović-Stanko et al., 2011). Extracorporeal shock wave lithotripsy and percutaneous nephrolithotomy are the two techniques used to diagnose this disease, but there is no evidence that these procedures will prevent stone development from returning (Geetha et al., 2010). Recently there has been a rising resurgence and revival of interest in traditional and indigenous medical practices, which are seen as being quite secure with few to no adverse effects, cost-effective, widely accessible, and easily attainable (Hayat et al., 2023; Hussain et al., 2022; Naveed et al., 2023). The use of *Ocimum* species in managing nephrotoxicity or issues related to urinary stones has been documented in an assortment of studies. An investigation of *O. basilicum* ethanol extract demonstrated nephron-protective action against cisplatin (Zaveri et al., 2011). If it refers to kidney stones, honey and the juice of basil leaves (*O. sanctum*) ingested routinely for six months will cause the stones to pass through the urinary tract (Kalyan et al., 2012). The management of urinary stones with a fresh infusion of *O. gratissimum* stem is documented in traditional medicine literature.

O. kilimandscharicum, also known as "Camphor Basil" in English and as "Kapoor Tulsi" in Hindi, is an atypical shrub that is primarily grown in South India. This plant is frequently used in traditional medicine to heal a broad range of conditions, including ulcers, abdominal discomfort, diarrhea, and microbial infection. Biologically active components in essential oils work as insect repellents, especially against mosquitoes and storage mites. The highest concentrations of camphor and oil are found in the leaves, which are followed by the blossoms. Sesquiterpenes in alcohol, terpinolene, d-pinene, d-limonene, and d-camphor are found in the stalks (Soni et al., 2012).

Literature review revealed the traditional use of various extracts of leaves and other parts of *Ocimum* species like *O. kilimandscharicum*, *O. basilicum*, *O. gratissimum* and *O. sanctum* for anti-urolithiasis (Nimavat et al., 2022), but no in vitro study has been undertaken for aerial parts of above mentioned *Ocimum* species for antiurolithiatic activity (Hussain et al., 2019). The presence of active constituents such as flavonoid and phenolic content in the extract of *Lepidagathis prostrata* whole plant (Devkar et al., 2016); terpenoids in *Bergenia ciliata* (Saha & Verma, 2013) and flavonoids, steroids, and terpenoids in *Bryophyllum pinnatum* (Nagarajan et al., 2019) were attributed to the antiurolithiatic activity. The present study aims to evaluate and compare the effectiveness of antiurolithiatic properties of extract and fractions of aerial parts of *O. kilimandscharicum*.

2. Materials and methods

2.1. Identification, authentication, and collection

The fresh aerial portions of OK were collected from Gandhi Krishi Vignan Kendra campus, Bangalore, Karnataka, India on 28/01/2020 and authenticated by Dr. Vijayakumar B. Narayanapur, College of Horticulture, Bagalkote, Karnataka, India. For reference in the future, the voucher specimen (NCP/01/2020-21) was stored in the herbarium of the Pharmacognosy Department at the National College of Pharmacy, Shimoga.

2.2. Extraction and fractionation procedure

Fresh aerial parts of OK were shade-dried for 10 days. The dried plant material was powdered and sieved through sieve no 44 to get the powder with a gritty texture. The weighed quantity (1 kg) of powdered plant material was extracted with 80% v/v hydro alcohol (5 l) using a round bottom flask for 4 days at room temperature by replacing fresh hydro alcohol daily replaced alcohol was collected, combined, and filtered. The filtrate was concentrated by using a rotary evaporator. 100 mg hydroalcoholic extract of *O. kilimandscharicum* (HAEOK) was further fractionated by using hexane and water (500 ml) as a solvent.

The hexane fraction was separated and evaporated. The aqueous layer was further partitioned using 50 ml of solvents such as chloroform, ethyl acetate, and *n*-butanol in 3 parts evaporated to dryness to get respective fractions (HFOK, AFOK, CFOK, EAFOK, and BFOK). The residual aqueous part was condensed by a rotary evaporator and finally dried at 60 °C. The dried extract/fractions were stored in the sealed container at 4 °C for the time being used (Hannan et al., 2006).

2.3. Preliminary phytochemical investigation

Standard qualitative tests were used to determine the phytoconstituents present in the HAEOK as per the procedures mentioned in the standard references (Khandelwal, 2008).

2.4. Total phenolic content

To assess the total phenolic content of the hydroalcoholic extract and its fractions of OK, the Folin-Ciocalteu reagent was used (Singleton & Rossi, 1965). In the extraction/fractions (100 mg/ml), 2.5 ml of 10% aluminum chloride solution and 2 ml of 7% sodium carbonate solution were used as neutralizing agents. The resulting reaction mixture was incubated for 30 min at room temperature leading to the development of a blue colour in the solution. The absorbance of the resulting blue-colored solution was measured at 765 nm. To estimate the phenolic content, a standard calibration curve of gallic acid was used (as the standard drug) and expressed in mg GAE per gram of dried extract/fraction.

2.5. Total flavonoid content

The concentration of total flavonoid content in hydroalcoholic extract and its fractions of OK was determined by using an aluminum chloride colorimetric assay (Chander et al., 2014). About

1 ml of extract/fractions (1 mg/ml) and 0.3 ml of sodium nitrate were added to the volumetric flask containing 4 ml of distilled water. Thereafter the solution was shaken well and it was kept aside for 5 min. After 5 min, 0.3 ml of 10% aluminum chloride was added and the volume was made up to 10 ml with distilled water and the solution was incubated for 30 min at room temperature. After mixing thoroughly, the absorbance of the solution was measured at 434 nm against a blank. The outcome of the present study was expressed as milligrams of quercetin (as a standard drug) dissolved in distilled water.

2.6. Investigation of in vitro antiurolithiatic property by titrimetry

2.6.1. Homogenous precipitation of calcium oxalate

A solution of calcium chloride dehydrate (1.47 gm) in 10 ml of 2 N sulphuric acid was allowed to react with sodium oxalate (1.34 gm) in distilled water. Calcium oxalate was precipitated and ammonia solution was used to remove traces of sulfuric acid from the solution. The precipitate was again rinsed with distilled water before being dried for 4 hours at 60 °C (Sathish & Jayebalan, 2017).

2.6.2. Synthesis of the semi-permeable membrane

Meanwhile, a semi-permeable membrane was prepared from the farm eggs by decalcifying the eggs in 2 M HCl for 24 hours until the complete removal of the shell part of the egg **(Figure 1)**. The decalcified egg was washed with distilled water and a hole was made carefully with a sharp pointer on the top and all the contents were squeezed out completely from the decalcified egg. Semi-permeable membrane was washed thoroughly with distilled water, and placed in ammonia solution, in the moistened conditions for a while, and then rinsed with distilled water. Finally, it is stored in the refrigerator at a pH of 7.0-7.4 until further need (Bhandari et al., 2021).

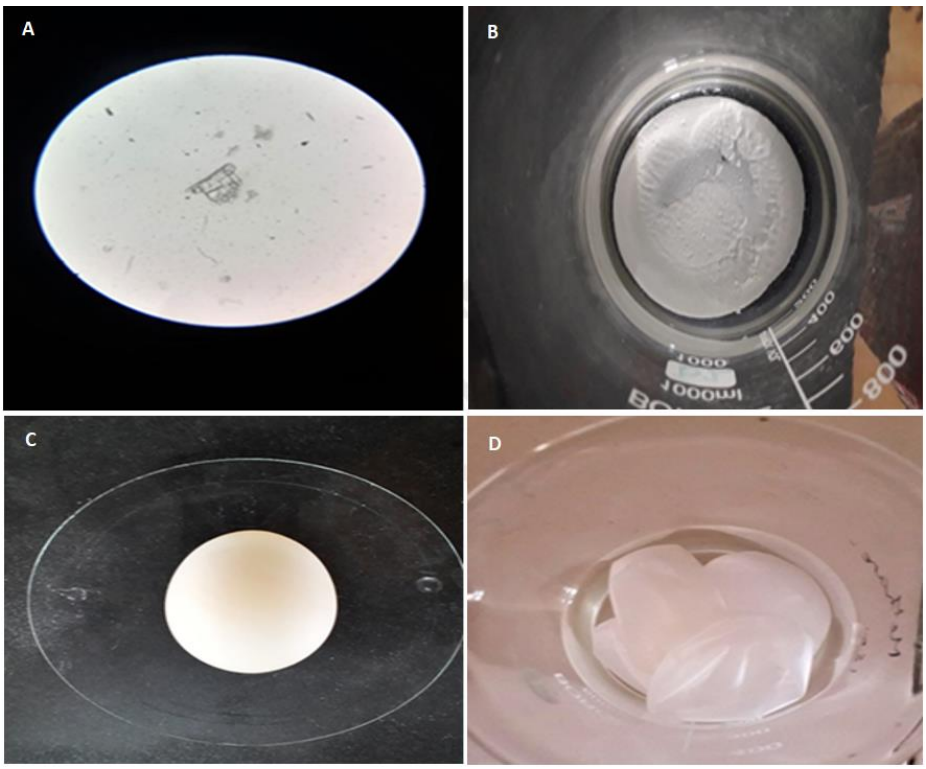

Figure 1. A) Microscopic view of calcium oxalate crystal, B) The homogenous precipitate of calcium oxalate, C) Decalcified egg, D) Semi-permeable membrane

2.6.3. Experimental method

In a semi-permeable membrane, 10 mg of the calcium oxalate and 100 mg of the sample/standard were weighed accurately and packed together. This was allowed to be suspended in a beaker containing 100 ml of 0.1 M TRIS buffer. Beakers of all groups were placed at room temperature at 37 °C for about 7-8 hours. The contents of semi-permeable were removed from each group into a test tube; 2 ml of 1 N H_2SO_4 was added and titrated against $KMnO_4$ till a light pink color endpoint was obtained. The amount of superfluous calcium oxalate is subtracted from the total quantity used at the beginning of the experiment. The percentage dissolution of calcium oxalate was calculated by using the factor 1 ml of 0.9494N $KMnO_4$ is equivalent to 0.1898 mg of calcium (Bhandari et al., 2021).

2.7. Calcium phosphate assay

2.7.1. Homogenous precipitation of calcium phosphate

An equimolar solution of disodium hydrogen phosphate (1.47 g) and calcium chloride (1.42 g) was prepared by dissolving in 2 N sulphuric acid and distilled water, respectively. Ammonia solution was used to wash out the traces of sulphuric acid from the resulting calcium phosphate precipitate. The crystals were rinsed in distilled water and dried for 4 hours at 60 °C (Sathish & Jayebalan, 2017).

2.7.2. Experimental method

Accurately weighed 10 mg of the calcium phosphate and 100 mg of extract/fractions/standard drug were packed together in a semipermeable membrane and sutured. The semipermeable membrane was prepared in the same manner as for the calcium

oxalate assay. This was allowed to be suspended in a conical flask containing 100 ml of 0.1 M TRIS buffer. All the conical flasks were placed uninterrupted at room temperature (37 °C) for 7-8 hours. The remaining contents in the semipermeable membrane were transferred into different test tubes, 4 ml of 1N H_2SO_4, 3 ml of molybdate-sulphuric acid reagent, and 1 ml of reducing solution were added to each test tube and kept aside for 2 hours; the color change was observed from dark pink to colorless. The optical densities were measured colorimetrically at 620 nm (Sathish & Jayebalan, 2017).

2.8. Statistical analysis

The outcomes of the experiment were expressed as mean ± SEM (n = 3). As per the suitability, a one-way ANOVA statistical method by using Graphpad Prism 9 software was used to evaluate the difference between the data. $p < 0.05$ was considered as significant and $p < 0.01$ was considered as very significant.

Table 1. Percentage yıeld of extract and varıous fractions of *O. kilimandscharicum*

Extract and fractions of OK	Percentage yield (%, w/w)
HAEOK	13.31 ± 0.02[a]
EAFOK	2.1 ± 0.01[a]
CFOK	1.2 ± 0.02[b]
BFOK	2.5 ± 0.02[a]
AFOK	2.2 ± 0.02[a]
HFOK	1.5 ± 0.03[b]

Percentage yield values are the mean of at least three replicates of experiments ± standard deviation; where a means $p < 0.05$, b means $p < 0.01$, sample vs standard in each group.

3. Results and discussion

The percentage yield of HAEOK, EAFOK, EAFOK, CFOK, BFOK, AFOK, and HFOK was 13.31 ± 0.02, 2.1 ± 0.01, 1.2 ± 0.02, 2.5 ± 0.02, 2.2 ± 0.02, and 1.5 ± 0.03, respectively **(Table 1)**. The preliminary phytochemical analysis of the extract reveals the fact that carbohydrates, saponins, flavonoids, steroids, triterpenoids, alkaloids, and phenolic compounds are present. Flavonoids and phenolic compounds are present in ethyl acetate fraction, triterpenoids in *n*-hexane fraction, alkaloids in the chloroform; carbohydrates, flavonoids, and phenolics in aqueous fraction; alkaloids and triterpenoids in *n*-butanol fraction **(Table 2)**. Flavonoids can prevent the adhesion of calcium oxalate crystals in the urinary tract due to its radical scavenging effect and stop further injury in the formation of kidney stones (Naveed et al., 2022a; Naveed et al., 2022b; Saleem et al., 2022). Saponins are known to disintegrate mucoproteins. Calcium oxalate crystal emergence and the dissolution of urinary stones that have already developed are inhibited by polyphenols and tannins (Bawari et al., 2018).

Table 2. Qualitative phytochemical analyses of the hydro-alcoholic extract of OK and its fractions

SL No	Chemical constituents	Test	EAFOK	CFOK	BFOK	AFOK	HFOK	HAEOK
1	Carbohydrates	Molish test	-	-	-	+	-	+
		Fehling test	-	-	-	+	-	+
		Benedict's test	-	-	-	+	-	+
2	Proteins	Ninhydrin test	-	-	-	-	-	-
		Xanthoproteic test	-	-	-	-	-	-
3	Alkaloids	Dragendroff's test	-	-	+	-	+	+
		Mayer's test	-	-	+	-	+	+
		Hager's test	-	-	+	-	+	+
		Wagner's test	-	-	+	-	+	+
4	Glycosides	Modified Brontrager's test	-	-	-	-	-	-
		Legal test	-	-	-	-	-	-
		Baljet test	-	-	-	-	-	-
5	Saponins	Foam test	-	-	-	+	-	+
6	Triterpenoids	Liebermann's Burchard's test	-	+	-	-	+	+
		Salkowski test	-	+	-	-	+	+
7	Flavonoids	Shinoda test	+	-	-	+	-	+
		Ferric chloride test	+	-	-	+	-	+
		Lead acetate test	+	-	-	+	-	+
8	Phenolics	Ferric chloride test	+	-	-	+	-	+

+ indicates present,- indicates absent

The presence of phenolic and flavonoid content was attributed to the medicinal effects of a wide range of angiosperms due to their positive pharmacological actions on human beings (Devkar et al., 2016). Polyphenols and flavonoids present in the plant extract could effectively inhibit the formation of calcium oxalate stones in vitro and in vivo, correlating with their diuretic, antioxidant, anti-inflammatory, antibacterial, and other protective effects (Ahmad et al., 2023; Monisa et al., 2023). *Persea americana* with high flavonoid content has been reported to have antilithiatic activity (Nagarajan et al., 2019). The percentage of phenolics and flavonoids was calculated using the aluminum chloride method and the Folin-Ciocalteu reagent method, respectively. The TPC and TFC of EAFOK were found to be higher when compared to other fractions (26.2 ± 0.03 mg GAE/g of fraction and 5.18 ± 0.03 mg QE/g of fraction, respectively). Quantitative estimation of the phenolics and flavonoids was carried out by calculating the percentage of phenolics and flavonoids altogether in the extract and its fractions of OK. TPC and TFC were computed in terms of gallic acid and quercetin equivalents out of each gram of extract/fractions depicted in **Table 3** respectively.

Table 3. TPC and TFC of hydro-alcoholic extract and its fractions of OK

Extract and fractions of OK	Percentage yield (%, w/w)	TFC (mg QE/g)	TPC (mg GAE/ g)
HAEOK	13.31 ± 0.02	8.18 ± 0.02[a]	30.5 ± 0.03 [a]
EAFOK	2.1 ± 0.01	5.18 ± 0.03[a]	26.2 ± 0.03 [a]
CFOK	1.2 ± 0.02	3.2 ± 0.02[a]	10 ± 0.06 [b]
BFOK	2.5 ± 0.02	4.2 ± 0.04[a]	15.6 ± 0.02 [a]
AFOK	2.2 ± 0.02	1.2 ± 0.02[b]	11.8 ± 0.02 [a]
HFOK	1.5 ± 0.03	2.5 ± 0.03[b]	8.5 ± 0.02 [b]

TPC and TFC values are the mean of at least three replicates of experiments ± standard deviation; where a means $p < 0.05$, b means $p < 0.01$, sample vs standard in each group.

Urolithiasis is a corporeal method of formation of calculi in the urinary tract (Ammara et al., 2023; Hussain et al., 2023). Oxidative stress in circumstances where stones develop is inevitable, even with advances in urology and nephrology. The structural integrity of membranes is compromised by oxalate, which causes lipid peroxidation. The by-product malondialdehyde is an important one. Antioxidant mechanisms in cells regulate lipid peroxidation; nonetheless, even with several antioxidant systems, overloading can lead to an increase in peroxidation products in tissues (Kant et al., 2020).

The concentration of Ca^{2+} in solutions was not proportionate to the dissolution of calcium oxalate. The effective percentage dissolution of calcium phosphate (59.03 ± 0.820) and calcium oxalate crystals (Figure 2) in the in-vitro antiurolithiatic investigation by HAEOK was found to be comparable to statistical studies conducted among all other fractions of OK and the standard drug cystone (48.03 ± 0.421%). The antiurolithiatic activity of the five fractions of OK's hydroalcoholic extract was assessed. With percent dissolution of calcium oxalate crystals CFOK, BFOK, AFOK, and HFOK were revealed to have considerable antiurolithiatic activity (39.21 ± 0.903%, 29.53 ± 0.414%, 21.82 ± 0.350%, 10.01 ± 0.312%, and 3.47 ± 0.670%, respectively) (Table 4).

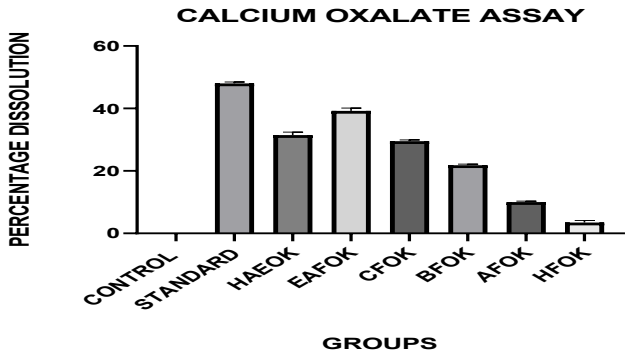

Figure 2. Calcium oxalate assay by titrimetry

The calcium phosphate calibration curve crystals at different concentrations were plotted against their respective optical densities as shown in Figure 3. The standard calibration curve of calcium phosphate was used to determine the undissolved calcium phosphate crystals. As a proportion dissolution, the study findings were analyzed. In calcium phosphate assay, HAEOK showed less significant antiurolithiatic activity than the standard drug with percentage dissolution of 59.03 ± 0.820% and 73.67 ± 0.453%, respectively (Tables 5 and 6). Among the various fractions of OK such as EAFOK was more significant than other fractions. But, it is less significant than cystone. The capability of percentage dissolution of calcium phosphate crystals by EAFOK, CFOK, BFOK, AFOK, and HFOK was found to be 66.62 ± 0.468%, 55.96 ± 0.436%, 44.67 ± 0.975%, 41.67 ± 0.642%, and 34.7 ± 0.983%, respectively (Figure 4). The EAFOK showed more significant antiurolithiatic activity but less significant than cystone drug among all other fractions of OK. HAEOK and EAFOK have a significant antiurolithiatic effect with significance levels of $p < 0.01$ and $p < 0.05$, respectively. The results of the present antiurolithiatic activity of hydroalcoholic extract and its fractions of *O. kilimandscharicum* were mainly ascribed to the inclusion of triterpenoids, phenols, and flavonoids. However, the data that has been obtained from the present investigation cannot simply provide an insight into the potential mechanism of action of tested phytoconstituents or extract. The phytochemical analysis of the extract reveals the presence of phenolics, flavonoids, triterpenoids, and other phytoconstituents in extract/fractions. The presence of these phytoconstituents may be responsible for antiurolithiatic activity. This medication can be used to treat urinary stone disease after additional study is done through in vivo experiments using experimental animals.

Table 3. TPC and TFC of hydro-alcoholic extract and its fractions of OK

Extract and fractions of OK	Percentage yield (%, w/w)	TFC (mg QE/ g)	TPC (mg GAE/g)
HAEOK	13.31 ± 0.02	8.18 ± 0.02[a]	30.5 ± 0.03 [a]
EAFOK	2.1 ± 0.01	5.18 ± 0.03[a]	26.2 ± 0.03 [a]
CFOK	1.2 ± 0.02	3.2 ± 0.02[a]	10 ± 0.06 [b]
BFOK	2.5 ± 0.02	4.2 ± 0.04[a]	15.6 ± 0.02 [a]
AFOK	2.2 ± 0.02	1.2 ± 0.02[b]	11.8 ± 0.02 [a]
HFOK	1.5 ± 0.03	2.5 ± 0.03[b]	8.5 ± 0.02 [b]

TPC and TFC values are the mean of at least three replicates of experiments ± standard deviation; where a means $p < 0.05$, b means $p < 0.01$, sample vs standard in each group.

4. Conclusions

The research outcome shows that HAEOK's antiurolithiatic action is less significant when compared to the standard. Comparing EAFOK to other fractions of OK, a very strong antiurolithiatic efficacy was observed. The extract's phytochemical assessment shows that it contains triterpenoids, flavonoids, phenolics, and other phytoconstituents. These phytoconstituents may be the cause of the antiurolithiatic action. Although *O. kilimandscharicum* has a wide range of biological uses, little scientific research has been done on the plant, and its full potential has not yet been realized. Furthermore, in vivo research has to be done on experimental animals; if more research is done, this medication may be utilized to treat urinary stone disease. Commercial use of *O. kilimandscharicum* for the production of medications intended to treat various illnesses.

Table 4. Impact of OK and its fractions on percentage dissolution of calcium oxalate crystals

Groups	Vol. of KMnO4 (ml)	Wt. of calcium estimated (mg)	Wt. of calcium reduced (mg)	% Dissolution
Control	4.2 ± 0.017	0.8034 ± 0.003	-	-
Standard**	2.2 ± 0.01	0.4176 ± 0.002	0.3859 ± 0.005	48.03 ± 0.421[a]
HAEOK**	2.9 ± 0.05	0.5504 ± 0.010	0.2531 ± 0.007	31.48 ± 0.920[a]
EAFOK**	2.6 ± 0.031	0.4884 ± 0.006	0.3151 ± 0.008	39.21 ± 0.903[a]
CFOK	3 ± 0.0153	0.5662 ± 0.003	0.2373 ± 0.004	29.53 ± 0.414[b]
BFOK	3.3 ± 0.027	0.6282 ± 0.005	0.1753 ± 0.002	21.82 ± 0.350[b]
AFOK	3.8 ± 0.027	0.7231 ± 0.005	0.0804 ± 0.002	10.01 ± 0.312[b]
HFOK	4.1 ± 0.015	0.7757 ± 0.003	0.0279 ± 0.005	3.47 ± 0.670[b]

Volume of $KMnO_4$ was measured as mean ± SD; where a means $p < 0.05$, b means $p < 0.01$, ($n = 3$).

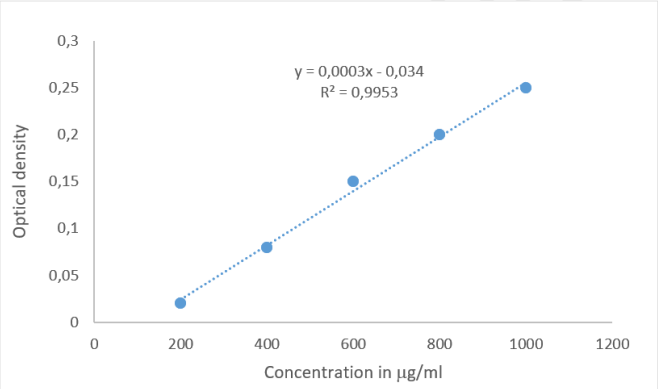

Figure 3. Calcium phosphate calibration curve

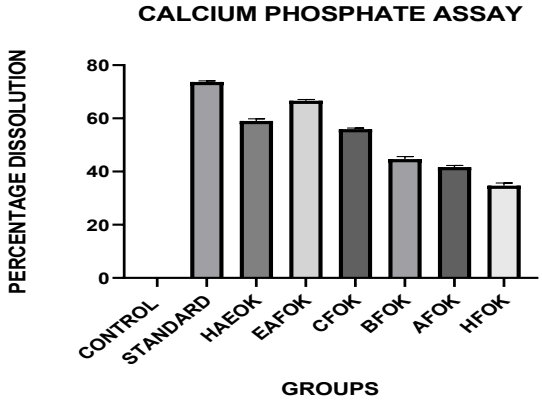

Figure 4. Calcium phosphate assay by colorimetry

Table 5. Standard calibration curve of calcium phosphate assay

Concentration (µg/ml)	Molybdic H_2SO_4 (ml)	Reducing agent (ml)	Distilled water (ml)	Optical density
200 µg/ml				0.02
400 µg/ml				0.08
600 µg/ml	2.5 ml	1 ml	q.s. to 10 ml	0.15
800 µg/ml				0.19
1000 µg/ml				0.25

Table 6. Calcium phosphate assay by colourimetry

Groups	Optical density	Calcium estimated (mg)	Calcium reduced (mg)	Percentage dissolution
Control	0.2483 ± 0.002	0.9849 ± 0.007	-	-
Standard**	0.0403 ± 0.002	0.2593 ± 0.005	0.7256 ± 0.006	73.67 ± 0.453[a]
HAEOK**	0.0817 ± 0.002	0.4035 ± 0.005	0.5814 ± 0.012	59.03 ± 0.820[a]
EAFOK**	0.060 ± 0.002	0.3349 ± 0.005	0.6561 ± 0.006	66.62 ± 0.468[a]
CFOK**	0.0903 ± 0.002	0.4337 ± 0.005	0.5511 ± 0.006	55.96 ± 0.436[a]
BFOK**	0.1217 ± 0.002	0.543 ± 0.005	0.4418 ± 0.11	44.67 ± 0.975[a]
AFOK*	0.1307 ± 0.002	0.5744 ± 0.007	0.4104 ± 0.007	41.67 ± 0.642[b]
HFOK*	0.1503 ± 0.002	0.643 ± 0.005	0.3418 ± 0.012	34.7 ± 0.983[b]

The optical densities were computed as mean ± SEM; where a means $p < 0.05$, b means $p < 0.01$, ($n = 3$)

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Conflict of interest

The authors confirm that there are no known conflicts of interest.

Statement of ethics

In this study, no method requiring the permission of the "Ethics Committee" was used.

Availability of data and materials

All data generated or analyzed during this study are included in this published article. On request, the associated author can provide more information.

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CRediT authorship contribution statement

Prathibha Guttal Subhas: Conceptualization, Investigation, Data curation, Writing-original draft, Supervision
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Supplementary File

None.

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