Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 66 Issue 06 | 2023
DOI 10.17605/OSF.IO/6U9XQ

INVESTIGATION OF THE ANTI-NEPHROLITHIAIS POTENTIAL OF BRYOPHYLLUM PINNALUM (Lam.) oken. LEAVES EXTRACTS/FRACTIONS IN INHIBITION OF CALCIUM OXALATE CRYSTALLIZATION

RATAN DEEP CHAUHAN

Amity Institute of Pharmacy, Amity University, Noida, Uttar Pradesh, India.
Email: ratan.chauhan@student.amity.edu

TANVEER NAVED*

Amity Institute of Pharmacy, Amity University, Noida, Uttar Pradesh, India.
*Correspondence Author Email: tnaved@amity.edu

MOHD. MUJEEB

School of Pharmaceutical Education & Research, Jamia Hamdard University, New Delhi, India.
Email: mmujeeb@jamiahamdard.ac.in

Abstract

The study's main objective is to evaluate the inhibitor effect of *Bryophyllum pinnatum* leaves extracts/fractions on the crystallization of calcium oxalate in synthetic urine. The study of calcium oxalate crystallization is based on changes in turbidity. The effect of *Bryophyllum pinnatum* leaves extracts/fractions on the crystallization of calcium oxalate was evaluated. Calcium oxalate crystals formation is caused by adding 10 to 50 mM sodium oxalate solution to synthetic urine. Adding extracts/fractions of *B. pinnatum* leaves (i.e., petroleum ether/chloroform/ethyl acetate/methanol/aqueous extract and ethyl acetate/ methanol fractions) allowed us to provide information on the inhibition percentage. The ethyl acetate and methanol extract/ ethyl acetate fractions-9,10 / methanol fractions-3,4 of *B. pinnatum* leaves displayed a maximum nucleation inhibition of 85.50, 91.03/73.62, 73.79/75.34, 77.24 percent observed at 10mg/ml and 5 mg/ml concentrations. The nucleation inhibition percentage of cystone is 57.24 at 10mg/ml. A potent dose-dependent aggregation inhibition was shown in the aggregation assay of ethyl acetate and methanol extract of *B. pinnatum* leaves, with a percentage inhibition of 74.62 and 84.7 percent. The ethyl acetate fractions-9, 10 with a percentage inhibition of 70.14 and 77.12 percent, and the methanol fractions-3, 4 with a percentage inhibition of 69.37 and 74.62 percent. In calcium oxalate nephrolithiasis, the ethyl acetate, methanol fractions, and ethyl acetate fractions-9, 10, methanol fractions-3, 4 of *B. pinnatum* leaves strongly inhibit crystal formation. The aggregation inhibition of cystone is 70.84.

Index Terms: *Bryophyllum pinnatum,* Nucleation, Aggregation, Calcium oxalate, Sodium oxalate

1. INTRODUCTION

1.1 Bryophyllum Pinnatum

The plant Bryophyllum pinnatum (Syn. Kalanchoe pinnata), belonging to the family Crassulaceae, is a native of tropical Africa [1]. Still, it has been naturalized throughout the tropics, particularly in India and Bengal. It is popularly known as Parnabija, Patharchuur, Patharchat, Hemsaagar (Ayurvedic) [2], Zakhm-Hayat (Unani) [3], Ranakkalli (Siddha),

Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 66 Issue 06 | 2023
DOI 10.17605/OSF.IO/6U9XQ

and Pathurkuchi in Bengali [4]. It is also known by other names such as Mother of Millions, Devil's Backbone, Pregnant Plant, Flopper, Air Plant, Miracle Leaf, Leaf of Life, Canterbury bells, Life Plant, Floppers, Good Luck Leaf, Mexican Love Plant, Tree of Life. In Brazil, the plant goes by the common name of saião or coirama; in Peru, it is called hoja del aire (air plant) or kalanchoe [5]. It is a perennial herb which is about 1m tall. Stem is fleshy and cylindrical, and the youngest stems are reddish. It is astringent and sour in taste [6]. It is classified as a weed [7]. *B. Pinnatum* is used medicinally in Indo-China and the Philippines Islands. It is naturalized throughout the hot and moist parts of India. The leaves are bitter tonic, analgesic, carminative, and useful in diarrhea and vomiting [8]. It is applied externally and taken internally for all pains, inflammations, bacterial, viral, and fungal infections, leishmaniasis, earaches, upper respiratory infections, stomach ulcers, flu, and fever [9]. In traditional medicine, the leaves of this plant have been used as antimicrobial[10],[11], antifungal[12], antiulcer (13), anti-inflammatory, analgesic[14],[15], antihypertensive[16], potent anti-histamine and anti-allergic activity[17], dysentery[18], headaches[19], for treating cancer symptoms[20],[21]. *B. Pinnatum* is rich in alkaloids, triterpenes, glycosides, flavonoids, cardenolides, steroids, bufadienolides, and lipids [22], [23], [24],]25]. The leaves contain a group of chemicals called bufadienolides which are very active. Bufadienolides like bryotoxin A, B, and C.

Fig. 1: *Bryophyllum pinnatum* leaves

1.2 Nephrolithiasis

Kidney stones affect all geographical, cultural, and racial groups. The lifetime risk is about 10-15% in the developed world but can be as high as 20-25% in the Middle East [26]. The number of deaths due to kidney stones is estimated at 19,000 per year, reasonably consistent between 1990 and 2010 [27]. In the United States, the frequency in the population of urolithiasis has increased from 3.2% to 5.2% from the mid-1970s to the mid-1990s [28]. In the United States, about 9% of the population has had a kidney stone. Kidney stone disease, also known as nephrolithiasis or urolithiasis, is a crystallopathy where a solid piece of material (kidney stone) develops in the urinary tract. Kidney stones typically form in the kidney and leave the body in the urine stream. A small stone may pass without causing symptoms. If a stone grows to more than 5 millimeters (0.2 inches), it can cause blockage of the ureter, resulting in sharp and severe pain in the lower back

Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 66 Issue 06 | 2023
DOI 10.17605/OSF.IO/6U9XQ

or abdomen [29]. A stone may also result in blood in the urine, vomiting, or painful urination. About half of those with a kidney stone will likely have another within ten years [30]. Most stones form by combining genetics and environmental factors. Risk factors include high urine calcium levels, obesity, certain foods, some medications, calcium supplements, hyperparathyroidism, gout, and not drinking enough fluids. Stones form in the kidney when minerals in urine are at high concentration. The diagnosis is usually based on symptoms, urine testing, and medical imaging. Blood tests may also be helpful. Stones are typically classified by their location: nephrolithiasis (in the kidney), ureterolithiasis (in the ureter), cystolithiasis (in the bladder), or by what they are made of (calcium oxalate, uric acid, struvite, cystine)

Pathogenesis of nephrolithiasis

It is understood that nephrolithiasis results from a combination of different mechanisms responsible for different types of stones.

Calcium stones: Calcium stones are the most common type of kidney stones [31]. Approximately 80% of all calcium stones are oxalate [32], [33]. The pathophysiology of calcium stones is complex and involves [34]: Dietary concerns, Hypercalciuria, Hypocitaturia, Hyperoxaluria, Hyperuricosuria, Biomineralization

Uric acid stones: The underlying pathophysiological mechanisms responsible for uric acid stones are Low urine volume, Hyperuricosuria, and High acidic urine.

Cystine stones: Cystinuria is a rare hereditary genetic disorder that causes impaired renal reabsorption of cationic amino acids and cystine. The cationic amino acids commonly involved are COLA, which stands for C= cystine, O=ornithine, L=lysine, and A=arginine.

Struvite/Ammonium magnesium sulfate stones: These are usually seen in patients who have infections with urease-positive organisms [35]. Urine pH is typically alkaline (>7.2).

2. MATERIALS AND METHODS

2.1 Collection of plant material and Authentication

Leaves of *Bryophyllum pinnatum* (Lam.) Oken was collected from the local herbal garden and authenticated by NISCAIR New Delhi, Reference No. NISCAIR/RHMD/Consult/2015/2922/115.

2.2 Extraction

Methanol crude leaves extract preparation

B. pinnatum leaves were washed clean, cut into small pieces with a Laboratory axe, shade-dried for 14 days in a well-ventilated place, and crushed with a blender. The crushed root materials were packed in airtight Ziploc plastics and stored in a refrigerator at four °C until required. The roots weighing 3kg were extracted using methanol solvent

Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 66 Issue 06 | 2023
DOI 10.17605/OSF.IO/6U9XQ

by continuous maceration using a magnetic stirrer for 72 hours. Then the extract was stippled and filtered. The Methanol crude extract was then dried in a vacuum at 40 ºC to obtain the powder whose yield was calculated. The extract was put in Ziploc containers and stored in the refrigerator at four °C until required.

Solvent partitioning fractionation

The methanol crude leaves extract powder was reconstituted with distilled water and partitioned with organic solvents in increasing order of polarity (petroleum ether, chloroform, ethyl acetate, and methanol) using a separating funnel.

Preparation of the petroleum ether fraction

To separate nonpolar phytochemical compounds from the crude extract, the extract was reconstituted with 200 ml of distilled water, partitioned with petroleum ether (3 x 200 ml), was agitated continuously for 2 minutes, and was allowed to settle for 60 minutes to form 2 layers. All nonpolar compounds, such as lipids and chlorophyll, were in the petroleum ether fraction (layer). This process is sometimes referred to as "defatting." Next, the petroleum ether fraction was dried under vacuum to powder.

Preparation of the chloroform fraction

The remaining aqueous suspension layer was partitioned three times with 200 ml chloroform, which was carried out by agitating the mixture continuously for 2 minutes. The mixture was allowed to settle for 60 minutes to form 2 layers. More minor polar phytochemical compounds were in the chloroform fraction, dried to a powder. The remaining second aqueous suspension layer was carried forward for the following procedure.

Preparation of the ethyl acetate fraction

The second aqueous suspension layer from the previous procedure was partitioned three times with 200 ml ethyl acetate. Partitioning was done by continuously agitating the mixture for 2 minutes and allowing it to settle for 60 minutes to give two layers. More minor polar phytochemical compounds were in the ethyl acetate fraction and dried to a powder. The remaining third aqueous suspension layer was carried forward for the following procedure.

Preparation of the methanol fraction

The third aqueous suspension layer from the previous procedure was partitioned with 200 ml methanol three times. Partitioning was done by agitating the mixture continuously for 2 minutes, after which it was allowed to settle for 60 minutes to form 2 layers. More polar phytochemical compounds were in the methanol fraction. The fraction was dried to a powder. The final aqueous suspension layer, containing the most polar phytochemical compounds in *B. pinnatum*, was dried to a powder.

Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 66 Issue 06 | 2023
DOI 10.17605/OSF.IO/6U9XQ

Silica gel filtration of *B. pinnatum* fractions

The potent fractions of *Bryophyllum pinnatum* were further fractionated by silica gel column chromatography to obtain sub-fractions. The Thin Layer Chromatography (T.L.C.) was done for each sub-fraction, and the sub-fractions with similar T.L.C. profiles (spots) were pooled.

Thin Layer Chromatography (T.L.C.)

The Thin Layer Chromatography was conducted on a glass sheet coated with a thin layer of adsorbent material, which, in this case, was silica gel impregnated with a fluorescent material. Each component on the T.L.C. appeared as spots separated vertically, each with a retention factor (R_f).

Extraction Procedure

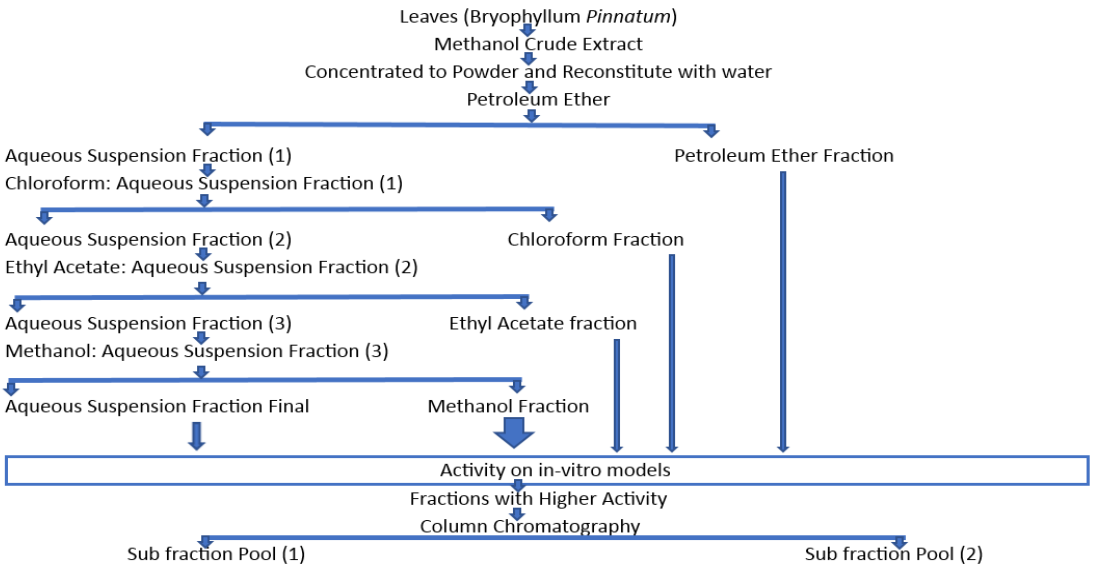

Fig 2: Extraction Procedure of *Bryophyllum Pinnatum* leaves

2.3 Phytochemical screening

Phytochemical screening for major constituents was undertaken using standard qualitative methods [36], [37]. The plant extracts were screened for alkaloids, flavonoids, steroids, terpenoids, phenol, carbohydrates, proteins, and amino acids.

2.4 Anticrystallization activity of extracts

The effect of the *B. pinnatum* leaves extracts/fractions and cystone on nucleation and aggregation of CaOx crystals was measured.

2.4.1 Nucleation assay

The inhibitory activity of the *B. pinnatum* leaves extracts/fractions/cystone on nucleation of CaOx crystals was determined based on the spectrophotometric assay [38].

Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 66 Issue 06 | 2023
DOI 10.17605/OSF.IO/6U9XQ

Crystallization was initiated by adding calcium chloride and sodium oxalate solutions to artificial urine. The artificial urine was prepared according to the method and had the following composition [39]. Sodium chloride-105.5 mmol/L. sodium phosphate-32.3 mmol/L. sodium citrate-3.21 mmol/L, magnesium sulfate-3.85 mmol/L, sodium sulfate-16.95 mmol/L, potassium chloride-63.7 mmol/L, calcium chloride-4.5 mmol/L, sodium oxalate-0.32 mmol/L, ammonium hydroxide-17.9 mmol/L, ammonium chloride-0.0028 mmol/L. The synthetic urine was prepared each day fresh and pH adjusted to 6.0.

The calcium chloride solution (4 mmol/L) and sodium oxalate solution (50 mmol/L) were prepared in a buffer containing tris 0.05 mol/L and NaCl 0.15 mol/L at pH 6.5 at 37ºC. The nucleation rate was determined by comparing the induction time of crystals (the time of appearance of crystals that have reached a critical size and thus become optically detectable) in the presence of extracts/fractions/cystone and that of the control without extracts/fractions/cystone. The control was made with sodium oxalate solution in distilled water at 10 to 50 mM (10, 20, 30, 40, and 50 mM) concentrations. The cystone solution was made in distilled water and filtered, and the concentration of 1, 2, 5, 7.5, and 10 mg/mL. The extract was prepared in distilled water and filtered, and a concentration of 10 mg/mL and fractions were prepared in distilled water, and a concentration of 5mg/ml was obtained. The absorbance was recorded at 620 nm spectrophotometrically. The percent inhibitions of plant extracts/fractions/cystone were calculated.

2.4.2 Aggregation assay

The CaOx crystals' aggregation rate was determined with slight modifications [40]. The CaOx crystals were prepared by mixing the calcium chloride solutions (4 mmol/L) and sodium oxalate (50 mmol/L). The CaOx crystals were then dissolved with tris 0.05 mol/L and NaCl 0.15 mol/L at pH 6.5 at a final 1 mg/mL concentration. The solutions were then incubated at 37^0C. Aggregation of CaOx crystals was determined in terms of change in absorbance which was recorded spectrophotometrically at 30, 60, 90, 180, and 360 minutes at 620 nm. The aggregation rate was estimated by comparing the absorbance in the presence of the plant extracts/fractions/cystone with that obtained in control. At the end of the treatment (360 min), the crystals were analyzed morphologically under the light microscope.

The percentage of inhibition was calculated using the formula: {1-[Si/Sc]} x100

Where; Si: change in absorbance per min in the presence of inhibitor (extracts/ fractions/cystone), Sc: change in absorbance per min of control (without inhibitor).

2.5 Statistical analysis

Results are expressed as mean ± SEM; n = 10. Values shown in parenthesis indicate percent inhibitions b compared to sodium oxalate treated and sodium oxalate + extracts/fractions treated. Level of significance *p < 0.05; **p < 0.01; ***p < 0.001.

Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 66 Issue 06 | 2023
DOI 10.17605/OSF.IO/6U9XQ

3. RESULTS AND DISCUSSION

3.1 Phytochemical screening

The phytochemical analysis of the methanolic extract of *B. pinnatum* leaves showed bioactive compounds in the plant, such as alkaloids, flavonoids, steroids, phenols, terpenoids, and proteins. Saponin was not detected.

Table 1: *Bryophyllum pinnatum* leaves Chemical tests on different extracts

S. No.	Type of Phyto-Constituents	Name of test	Extract(s)				
			Pet. Ether	Chloroform	Ethyl acetate	Methanol	Aqueous
1	Alkaloids	a. Mayer test	-	+	-	++	-
		b. Wagner test	-	+	+	++	-
		c. Dragendroff's test	-	-	+	+	-
2	Carbohydrates	a. Molisch test	-	-	-	-	-
		b. Fehling test	-	-	-	-	-
3	Steroids	a. Liebermann test	+	-	+	+	-
		b. Salkowski test	+	+	++	+ +	-
		a. Vanadate test	-	-	-	+	-
4	Triterpenoids	b. Salkowski test	-	-	+	+	+
5	Flavonoids	a. Mineral acid test	-	+	+	+	+
5	Flavonoids	b. Sod. Hydroxide	-	-	+	+	+
6	Saponins	c. Shinoda test	+	-	+	+	+
		Froth formation test	-	-	-	-	-
7	Tannins	a. Ferric chloride test	-	-	-	-	-
7	Tannins	b. Acetic acid test	-	-	-	-	-
8	Cardenolides	Keller Killiani	+	+	-	+	-

3.2 Anticrystallization activity of extracts

In this study, an in-vitro inhibitory effect of various extracts/fractions of *B. pinnatum* leaves (i.e., petroleum ether/chloroform/ethyl acetate/methanol/aqueous extract and ethyl acetate/ methanol fractions) on various phases of calcium oxalate crystallization was determined by time course measurement of turbidity in the synthetic urine at graded concentrations and compared with standard polyherbal drug cystone. The results revealed that the addition of sodium oxalate (10-50 mM) in artificial urine induced the formation of calcium oxalate crystals by significantly increasing the nucleation and aggregation rate of the construction of calcium oxalate crystals in a concentration and time-dependent manner, as in Table 2. The significant initial increase in absorbance till 30 min of incubation of sodium oxalate with artificial urine corresponds to the nucleation of the calcium oxalate crystals and is then followed by a decrease in absorbance, indicating the aggregation rate of the crystals as in Fig 3. Table 4, 5, 6 presents the mitigatory effect of the *Bryophyllum pinnatum* leaves extracts/fractions on sodium oxalate-induced crystallization in artificial urine.

3.2.1. Nucleation assay

In nucleation assay (0-30 min), the number of crystals formed was estimated in terms of the turbidity of the solution. The absorbance of the control recorded was subtracted from that obtained with the *B. pinnatum* leaves extracts/fractions. There is a steep decrease in the absorbance with the *B. pinnatum* leaves extracts/fractions and cystone when

Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 66 Issue 06 | 2023
DOI 10.17605/OSF.IO/6U9XQ

incubated with sodium oxalate (50 mM). The percentage inhibition of *B. pinnatum* leaves extracts was (petroleum ether-30.68/chloroform- 46.37/ethyl acetate- 85.50/methanol-91.03/aqueous- 61.72), acetate fractions (BPEA-1.-44.31, BPEA-2.-44.31, BPEA-3.-35.86, BPEA-4.-30.51, BPEA-5.-41.55, BPEA-6.-47.58, BPEA-7.-56.20, BPEA-8.-65.00, BPEA-9.-73.62, BPEA-10.-73.79) and methanol fractions (BPME-1.-47.58, BPME-2.-49.65, BPME-3.-75.34, BPME-4.-77.24, BPME-5.-58.96, BPME-6.-47.75, BPME-7.-56.37, BPME-8.-65.00, BPME-9.-36.72, BPME-10.-30.51). However, cystone showed percent inhibition in 17.75-57.24 percent, as in Table 3 and Fig.4.There was a dose-dependent increase in the percent inhibition of nucleation by the extracts/fractions of *B. pinnatum* and cystone. The IC50 value of *B. pinnatum* ethyl acetate and methanol extracts/ ethyl acetate (BPEA-9, 10)/ methanol (BPEA-3, 4) fractions were found to be less IC-50 value (more potent) than the cystone with an IC-50 value of 8.1 mg/mL.

3.2.2. Aggregation assay

Similarly, in aggregation assay, *Bryophyllum pinnatum* leaves extracts/fractions showed more significant concentration-dependent inhibition of the aggregation of crystals of CaOx when compared with cystone as in Table 3. However, cystone also showed inhibitory activity on crystal aggregation but is comparatively less potent than the *Bryophyllum pinnatum*

Leaves extracts (petroleum ether-38.26-42.50/chloroform- 49.44-56.21/ethyl acetate-63.66-74.62/methanol- 71.66-91.88/aqueous- 57.50-64.67), acetate fractions (BPEA-1.-43.33-44.69, BPEA-2.-43.61-54.96, BPEA-3.-32.77-62.36, BPEA-4.-35.27-61.99, BPEA-5.-37.37-61.62, BPEA-6.-40.55-65.31, BPEA-7.-50.00-63.66, BPEA-8.-57.50-63.34, BPEA-9.-60.55-70.41, BPEA-10.-63.33-77.12) and methanol fractions (BPME-1.-32.50-43.91, BPME-2.-32.50-48.75, BPME-3.-57.50-69.37, BPME-4.-60.27-74.62, BPME-5.-38.05-63.66, BPME-6.-40.55-66.88, BPME-7.-48.88-66.88, BPME-8.-57.50-66.88, BPME-9.-32.50-62.336, BPME-10.-35.83-62.36) at the same concentration range of 10mg/ml and 5mg/ml, as in Fig.5,6,7. The percent inhibitions at 360 min of the marketed drug, cystone, were 19.44-70.84 with an IC50 value of 3.26 mg/mL as in Fig.4. The IC50 value of the ethyl acetate and methanol extracts *of B. pinnatum* was 1.107 and 0.9 mg/mL, respectively. The coefficient of regression *r2* was obtained by linear regression. All results exhibited a regression coefficient with r2 > 0.9 ($p < 0.01$).

Table 2: Effect of sodium oxalate on calcium oxalate crystallization in artificial urine

S. No.	Experimental groups	Nucleation	Aggregation			
		Change in absorbance/min.				
		0-30th min	31-60th min	61-90th min	91-180th min	181-360th min
Sodium oxalate (NaOx) (mM) - Treated						
1	NaOx (10)	0.187±0.0015	0.089±0.0010	0.078±0.0013	0.050±0.0014	0.033±0.0038
2	NaOx (20)	0.268±0.0013	0.130±0.0014	0.084±0.0026	0.060±0.0014	0.039±0.0017
3	NaOx (30)	0.349±0.0017	0.200±0.0014	0.128±0.0013	0.102±0.0013	0.070±0.0014
4	NaOx (40)	0.461±0.0017	0.314±0.0026	0.248±0.0013	0.203±0.0021	0.163±0.0021
5	NaOx (50)	0.580±0.0014	0.360±0.0014	0.311±0.0010	0.271±0.0010	0.201±0.0010

Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 66 Issue 06 | 2023
DOI 10.17605/OSF.IO/6U9XQ

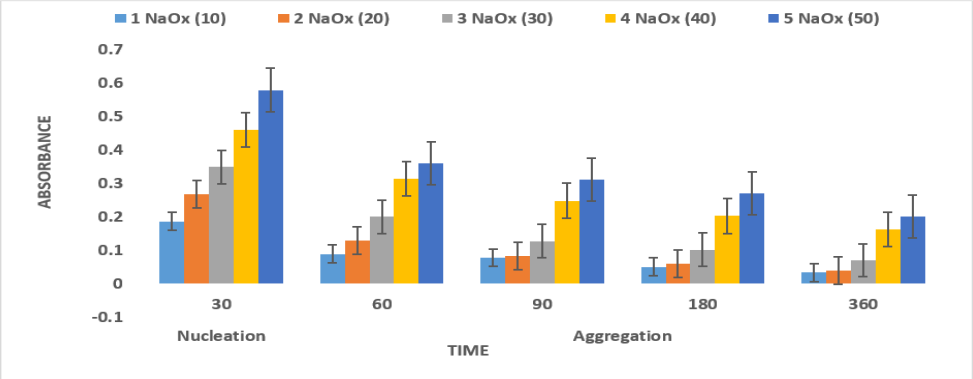

Fig 3: Effect of different concentrations of sodium oxalate (mM) on calcium oxalate crystallization in artificial urine

Table 3: Effect of sodium oxalate and cystone on calcium oxalate crystallization in artificial urine

S. No.	Experimental groups	Nucleation	Aggregation			
		Change in absorbance/min.				
		0-30th min	31-60th min	61-90th min	91-180th min	181-360th min
Sodium oxalate (NaOx) (mM) + Cystone (CST) (mg/ml) – Treated.						
1.	NaOx (50) + CST (1)	0.477±0.0021 b(17.75)	0.290±0.0014 b(19.44)	0.241±0.0017 b(22.50)	0.205±0.0026 b(24.35)	0.142±0.0013 b(29.35)
2.	NaOx (50) + CST (2)	0.412±0.0013 b(28.96)	0.262±0.0013 b(27.22)	0.213±0.0021 b(31.51)	0.168±0.0024 b(38.00)	0.121±0.0017 b(39.80)
3.	NaOx (50) + CST (5)	0.372±0.0013 b(35.86)	0.212±0.0013 b(41.11)	0.172±0.0013 b(44.69)	0.144±0.0022 b(46.86)	0.102±0.0013 b(49.25)
4.	NaOx (50) + CST (7.5)	0.323±0.0021* b(44.31)	0.182±0.0013* b(50.00)	0.152±0.0013* b(51.12)	0.132±0.0013* b(51.29)	0.092±0.0013* b(54.22)
5.	NaOx (50) + CST (10)	0.248±0.0013* b(57.24)	0.158±0.0013* b(56.11)	0.132±0.0013* b(57.55)	0.079±0.0023* b(70.84)	0.068±0.0024* b(66.16)

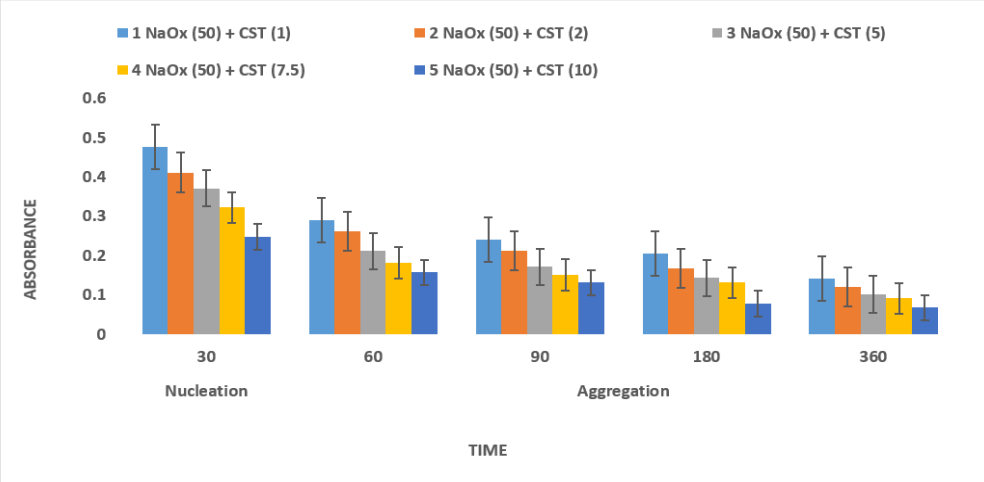

Fig 4: Effect of various concentrations of cystone (mg/mL) and sodium oxalate (50 mM) on calcium oxalate crystallization

Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 66 Issue 06 | 2023
DOI 10.17605/OSF.IO/6U9XQ

Table 4: Effect of sodium oxalate and *Bryophyllum pinnatum* (BP) extracts on calcium oxalate crystallization in artificial urine

S. No.	Experimental groups	Nucleation	Aggregation			
		Change in absorbance/min.				
		0-30th min	31-60th min	61-90th min	91-180th min	181-360th min
Sodium oxalate (NaOx) (mM) + *Bryophyllum pinnatum* (BP) extracts (10mg/ml)- Treated						
1.	NaOx (50) + BPPE (10)	0.402±0.0013 b(30.68)	0.207±00.0021 b(42.50)	0.192±0.0013 b(38.26)	0.163±0.0021 b(39.85)	0.122±0.0020 b(39.30)
2.	NaOx (50) + BPCH (10)	0.311±0.0023* b(46.37)	0.182±0.0013* b(49.44)	0.153±0.0015* b(50.80)	0.120±0.0014* b(55.71)	0.088±0.0013* b(56.21)
3.	NaOx (50) + BPEA (10)	0.084±0.0016** b(85.50)	0.132±0.0013** b(63.33)	0.092±0.0013** b(70.41)	0.072±0.0013** b(73.43)	0.051±0.0010** b(74.62)
4.	NaOx (50) + BPME (10)	0.052±0.0013** b(91.03)	0.102±0.0013** b(71.66)	0.072±0.0013** b(76.84)	0.052±0.0013** b(91.88)	0.032±0.0013** b(84.07)
5.	NaOx (50) + BPAQ (10)	0.222±0.0013* b(61.72)	0.153±0.0013* b(57.50)	0.123±0.0015* b(60.45)	0.102±0.0013* b(55.71)	0.071±0.0010* b(64.67)

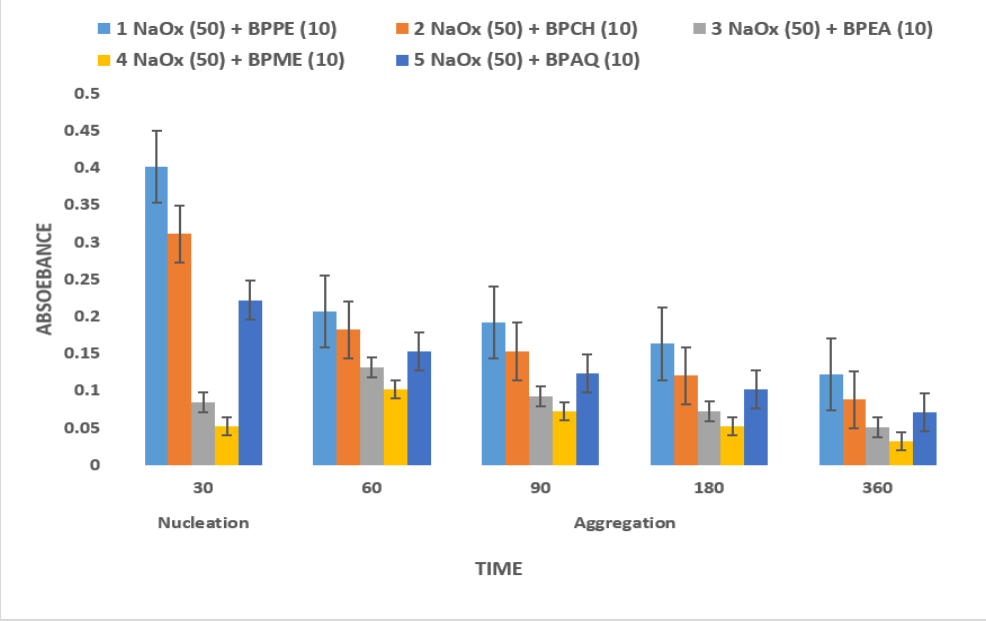

Fig 5: Effect of various extracts of *Bryophyllum pinnatum* (mg/mL) and sodium oxalate (50 mM) on calcium oxalate crystallization in artificial urine

Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 66 Issue 06 | 2023
DOI 10.17605/OSF.IO/6U9XQ

Table 5: Effect of sodium oxalate and Bryophyllum *pinnatum* (BP) ethyl acetate fractions on calcium oxalate crystallization in artificial urine

S. No.	Experimental groups	Nucleation	Aggregation			
		Change in absorbance/min.				
		0-30th min	31-60th min	61-90th min	91-180th min	181-360th min
Sodium oxalate (NaOx) (mM) + *Bryophyllum pinnatum* (BP) ethyl acetate fractions (10mg/ml)- Treated						
1.	NaOx (50) + BPEA-1 (5)	0.323±0.0015 b(44.31)	0.204±0.0022 b(43.33)	0.172±0.0013 b(44.69)	0.162±0.0013 b(40.22)	0.112±0.0013 b(44.27)
2.	NaOx (50) + BPEA-2 (5)	0.323±0.0015 b(44.31)	0.203±0.0015 b(43.61)	0.142±0.0013 b(54.34)	0.122±0.0013 b(54.96)	0.102±0.0013 b(49.25)
3.	NaOx (50) + BPEA-3 (5)	0.372±0.0013 b(35.86)	0.242±0.0013 b(32.77)	0.132±0.0013 b(57.55)	0.102±0.0013 b(62.36)	0.092±0.0013 b(54.22)
4.	NaOx (50) + BPEA-4 (5)	0.403±0.0015 b(30.51)	0.233±0.0015 b(35.27)	0.133±0.0015 b(57.23)	0.103±0.0015 b(61.99)	0.093±0.0015 b(53.73)
5.	NaOx (50) + BPEA-5 (5)	0.339±0.0017 b(41.55)	0.224±0.0022 b(37.37)	0.123±0.0021 b(60.45)	0.104±0.0021 b(61.62)	0.094±0.0022 b(53.23)
6.	NaOx (50) + BPEA-6 (5)	0.304±0.0022* b(47.58)	0.214±0.0022* b(40.55)	0.124±0.0022* b(60.12)	0.094±0.0022* b(65.31)	0.084±0.0022* b(58.20)
7.	NaOx (50) + BPEA-7 (5)	0.254±0.0022* b(56.20)	0.184±0.0069* b(50.00)	0.113±0.0015* b(63.66)	0.102±0.0015* b(62.36)	0.082±00.0013* b(59.20)
8.	NaOx (50) + BPEA-8 (5)	0.203±0.0015* b(65.00)	0.153±0.0015* b(57.50)	0.114±0.0022* b(63.34)	0.102±0.0022* b(62.36)	0.083±0.0015* b(58.70)
9.	NaOx (50) + BPEA-9 (5)	0.1533±0.0015* *b(73.62)	0.142±0.0013** b(60.55)	0.092±0.0013** b(70.41)	0.082±0.0013** b(69.74)	0.072±0.0013** b(64.17)
10.	NaOx (50) + BPEA-10 (5)	0.152±0.0013** b(73.79)	0.132±0.0013** b(63.33)	0.082±0.0013** b(73.63)	0.062±0.0013** b(77.12)	0.062±0.0013** b(69.15)

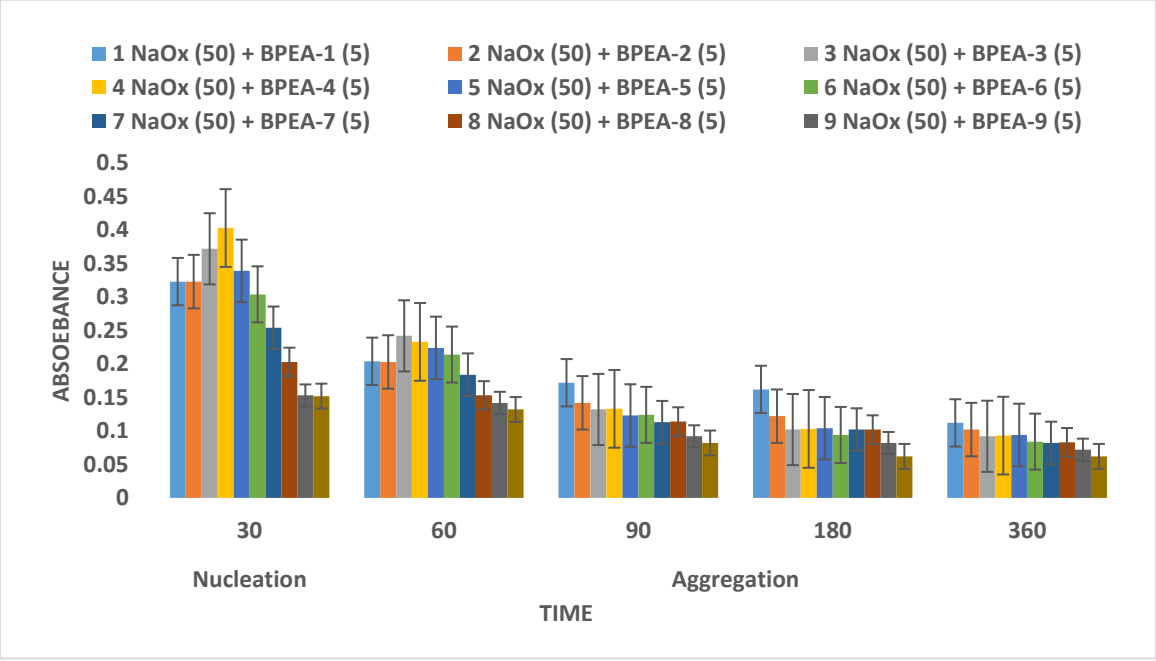

Fig 6: Effect of various Ethyl acetate fractions of *Bryophyllum pinnatum* (mg/mL) and sodium oxalate (50 mM) on calcium oxalate crystallization in artificial urine

Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 66 Issue 06 | 2023
DOI 10.17605/OSF.IO/6U9XQ

Table 6: Effect of sodium oxalate and Bryophyllum *pinnatum* (BP) methanol fractions on calcium oxalate crystallization in artificial urine

S. No.	Experimental groups	Nucleation	Aggregation			
		Change in absorbance/min.				
		0-30th min	31-60th min	61-90th min	91-180th min	181-360th min
Sodium oxalate (NaOx) (mM) + *Bryophyllum pinnatum* (BP) methanol fractions (10mg/ml)- Treated						
1.	NaOx (50) + BPME-1 (5)	0.304±0.0221 b(47.58)	0.243±0.0153 b(32.50)	0.193±0.0153 b(37.94)	0.152±0.0133 b(43.91)	0.113±0.0153 b(43.78)
2.	NaOx (50) + BPME -2 (5)	0.292±0.0200 b(49.65)	0.243±0.0153 b(32.50)	0.182±0.0133 b(41.47)	0.152±0.0133 b(43.91)	0.103±0.0153 b(48.75)
3.	NaOx (50) + BPME -3 (5)	0.143±0.0153** b(75.34)	0.153±0.0153** b(57.50)	0.103±0.0213** b(66.88)	0.083±0.0153** b(69.37)	0.062±0.0133** b(69.15)
4.	NaOx (50) + BPME -4 (5)	0.132±0.0133** b(77.24)	0.143±0.0213** b(60.27)	0.103±0.0213** b(66.88)	0.072±0.0133** b(73.43)	0.051±0.0100** b(74.62)
5.	NaOx (50) + BPME -5 (5)	0.342±0.0133 b(58.96)	0.223±0.0213 b(38.05)	0.113±0.0213 b(63.66)	0.104±0.0306 b(61.62)	0.081±0.0100 b(59.70)
6.	NaOx (50) + BPME -6 (5)	0.303±0.0213* b47.75)	0.214±0.0221* b(40.55)	0.103±0.0213* b(66.88)	0.093±0.0153* b(65.68)	0.083±0.0153* b(58.70)
7.	NaOx (50) + BPME -7 (5)	0.253±0.0153* b(56.37)	0.184±0.0163* b(48.88)	0.103±0.0153* b(66.88)	0.091±0.0100* b(66.42)	0.081±0.0100* b(59.70)
8.	NaOx (50) + BPME -8 (5)	0.203±0.0153* b(65.00)	0.153±0.0153* b(57.50)	0.103±0.0213* b(66.88)	0.092±0.0133* b(66.05)	0.072±0.0133* b(64.17)
9.	NaOx (50) + BPME -9 (5)	0.367±0.0153 b(36.72)	0.243±0.0153 b(32.50)	0.132±0.0133 b(57.55)	0.102±0.0133 b(62.36)	0.092±0.0133 b(54.22)
10.	NaOx (50) + BPME -10 (5)	0.403±0.0153 b(30.51)	0.231±0.0180 b(35.83)	0.121±0.0180 b(61.09)	0.102±0.0133 b(62.36)	0.087± 0.0153 b(56.71)

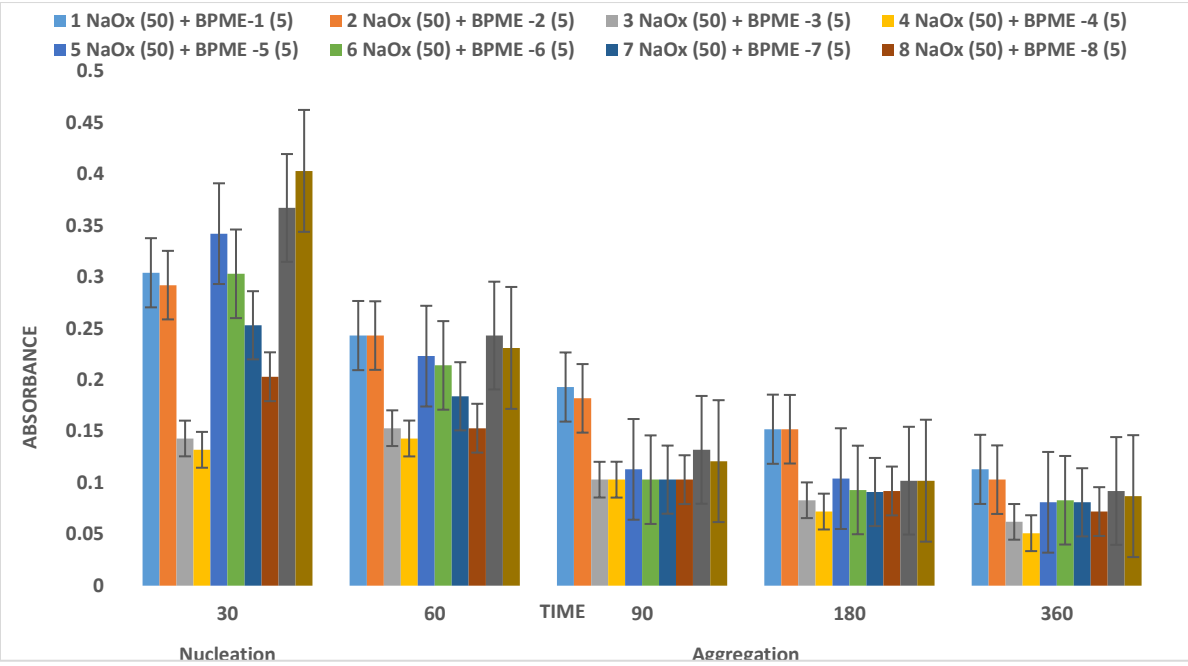

Fig 7: Effect of various Methanol fractions of *Bryophyllum pinnatum* (mg/mL) and sodium oxalate (50 mM) on calcium oxalate crystallization in artificial urine.

Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 66 Issue 06 | 2023
DOI 10.17605/OSF.IO/6U9XQ

3.3. Microscopic analysis

The light microscopic photographs taken at 360 min of sodium oxalate (50 mM) treatment in artificial urine showed the formation of both types of CaOx crystals, oval-shaped calcium oxalate monohydrate (COM) and bipyramidal-shaped calcium oxalate dehydrate (COD) with significant aggregations (Fig.8F). However, petroleum ether/chloroform/ethyl acetate/methanol/aqueous extract/fractions of *B.* pinnatum at 10 mg/mL/5 mg/mL concentration caused inhibition in the CaOx crystal formation with no COM crystals (Fig. 8A,7B,7C,7D, and 7E). Moreover, the number of COD crystals was also less with ethyl acetate (BPEA-9, 10)/methanol(BPEA-3, 4) fractions of *B.* pinnatum. Furthermore, the marketed drug cystine's results were less significant than the ethyl acetate and methanol extracts/ ethyl acetate(BPEA-9, 10)/methanol (BPEA-3, 4) fractions with numerous COM and COD crystals at the same concentration level Thus, the ethyl acetate and methanol extract methanol extracts/ ethyl acetate(BPEA-9, 10)/methanol (BPEA-3, 4) fractions of *B. pinnatum* were more potent than cystone, as depicted by the IC50 values in nucleation and aggregation assays and light microscopy photographs.

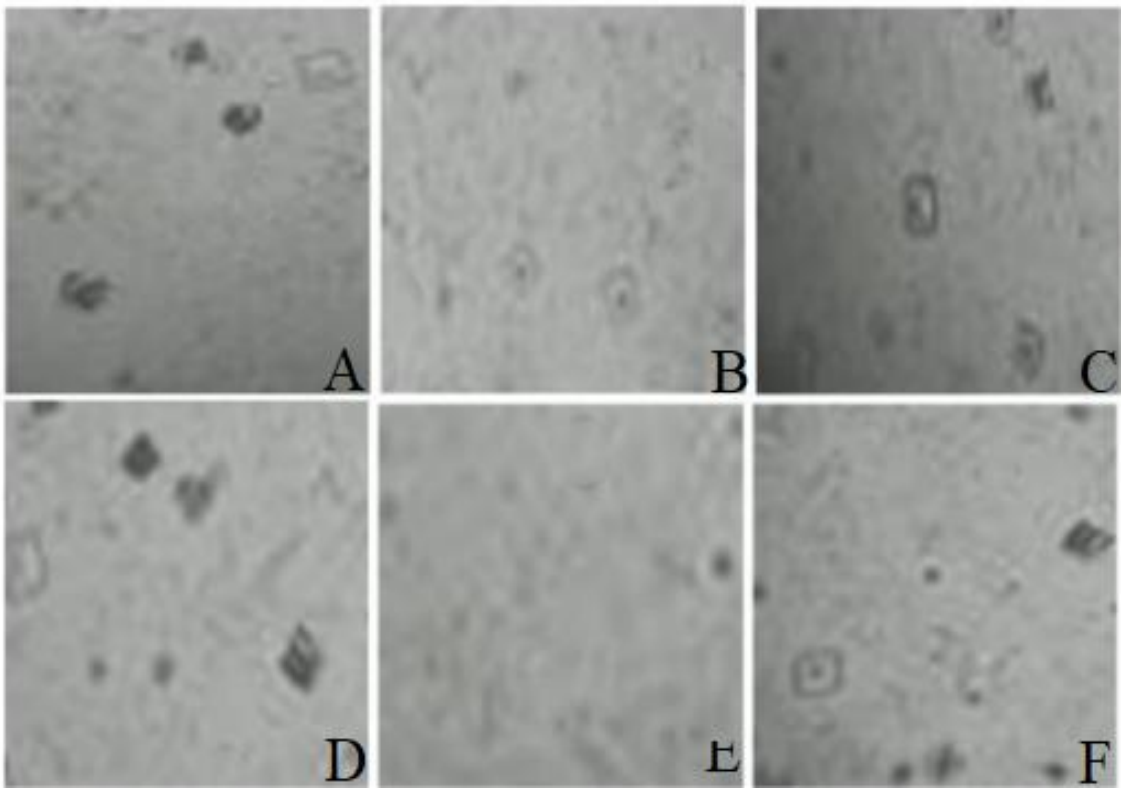

Fig 8: Nucleation and aggregation (A) Pet ether extract (10 mg/mL) (B) Chloroform Extract (10 mg/mL) (C) Ethyl acetate Extract (10 mg/mL) (D) Methanol Extract (10 mg/mL) (E) Cystone (10 mg/mL) along with sodium oxalate (50 mM) (F) Sodium oxalate (50 mM).

Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 66 Issue 06 | 2023
DOI 10.17605/OSF.IO/6U9XQ

4. CONCLUSION

Natural drugs are proven to target only one aspect of nephrolithiasis pathophysiology, whereas herbal medications have been shown to exert effectiveness at different stages of stone pathophysiology. Herbal medicines produce multiple mechanisms of action and, therefore, might have crystallization inhibition activity in addition to inhibition of stone formation. In the present study, the plant extracts/fractions significantly inhibit nucleation and aggregation of calcium oxalate crystallization in vitro. The microscopic photographs also showed a reduced number and size of calcium oxalate crystals by the plant extracts/fractions in a dose-dependent manner. Crystal aggregation is the most critical step as it occurs very fast and considerably affects particle size, and aggregated crystals are commonly found in urine and renal stones. *B. pinnatum* has several polyphenolic constituents like alkaloids, flavonoids, saponins, terpenoids, and tannins. Saponins are known to possess anticrystallization properties by disaggregating the suspension of mucoproteins, promoters of crystallization.

Acknowledgment

The authors thank the Amity Institute of Pharmacy, Amity University, Noida, for providing research facilities.

References

1. Anonymous (1998), 'The Wealth of India-An Encyclopedia of India's Raw Material Series,' CSIR-NIScPR, New Delhi, Vol. X, pp. 588-590.
2. Nagaratna A, Prakash L Hegde. A comprehensive review on parnabeeja (*Bryophyllum pinnatum* (lam.) Oken). 2015 Aug 29; 6.
3. Kirtikar & Basu, "Indian medicinal Plants," vol.2, published by Lalit Mohan Basu, pp. 997-1002.
4. Sofowora A. Medicinal plants and traditional medicine in Africa. 2nd ed. Ibadan. Polygraphic Ventures. Ltd.; 1993. p. 207-9.
5. L.S. Gill (1992). Ethno-medical uses of the plant in Nigeria: UNIBEN Press. 46:
6. Prasad AK, Kumar S, Iyer SV, Sudani RJ, Vaidya SK. Pharmacognostical, Phytochemical and Pharmacological Review on *Bryophyllum pinnatum*. 2010; 3:11.
7. B. Oliver-Bever (1983). Medicinal plants in tropical West Africa-III Antiinfection therapy with higher plants. J. Ethno pharmacology. 9: 1-83.
8. K.R. Kirtikar, and B.D. Basu. Indian Medicinal Plants, periodical experts. Delhi. 2: 2nd ed: 999 (1975).
9. S.A. Da Silva, S.S. Costa, S.C. Mendonca, E.M. Silva, V.L. Moraes, and B. Rossi Bergmann. The therapeutic effect of oral *Kalanchoe Pinnata* leaf extract in murine leishmaniasis. Acta Tropica. 60 (3): 201-210(1995).
10. Mehta and J.U. Bhat. Studies on Indian Medicinal plant II, podophyllin, a new antibacterial substance from leaves of Bryophyllum, Calycinum Salsib. J University Bombay. 21: 21-25(1952).
11. D.A. Akinpelu. Antimicrobial activity of *Bryophyllum Pinnatum* leaves Fitoterapia—71 (2): 193-194 (2000).
12. S. Misra and S.N. Dixit. Antifungal activity of leaf extract of some higher plants. Acta Botanica Indica. 7: 147-150 (1979).

ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 66 Issue 06 | 2023
DOI 10.17605/OSF.IO/6U9XQ

13. S. Pal and A.K. Nag Chaudhari. Studies on the Anti-Ulcer activity of a *Bryophyllum Pinnatum* leaf extract in experimental animals. J. Ethnopharmacol. 33: 97-102(1991).
14. S. Pal and A.K. Nag Chaudhari. Preliminary studies on the anti-inflammatory and analgesic activities of *Bryophyllum pinnatum*. Med Sci Res. 17: 561-562(1989).
15. S. Pal and A.K. Nag Chaudhari. Further studies on the anti-inflammatory profile of the methanolic fraction of the fresh leaf extract of *Bryophyllum pinnatum*. Fitoterapia. 63: 451-459(1992).
16. J.A.O. Ojewole. Antihypertension properties of *Bryophyllum pinnatum* (Lam) (oken) leaf extracts. Am J. Hypert. 15(4): A34-A39 (2002).
17. S. Pal, T. Sen, and A.K. Nag Chaudhari. Neuro-psycho-pharmacological profile of the methanolic fraction of *Bryophyllum Pinnatum* leaf extract. J. Pharm. Pharmacol. 51: 313-318(1999).
18. J.M. Dalziel. The useful plants of west tropical Africa. London: Crown Agents for the Colonies. 28-29(1937).
19. A. Sofowora. Medicinal plants and traditional medicine in Africa. New York: John Wiley and Sons. 119 (1993).
20. P. Paranjpe. Indian Medicinal Plants Forgotten Healers. Chaukhamba Sanskrit Pratisthan, Delhi. 194-195(2005).
21. P.J. Mathew and M.C. Unithan. Search for plants having anticancer properties used by the tribals of Wyandu, Malappuram, and Palghat districts of Kerala. Indian Aryavaidan. 6: 54-60(1992).
22. P.B. Marriage and D.G. Wilson. Analysis of Organic acids of *Bryophyllum pinnatum*. Can. J. Biochem. 49: 282-295(1971).
23. K. Gaind and R. Gupta. Alkanes, Alkanols, Triterpenes, and Sterols of Kalanchoe pinnata. Phytochemistry. 11: 1500-1502 (1972).
24. K. Gaind and R. Gupta. Identification of waxes from leaves of Kalanchoe pinnata. Planta Medica. 23: 193-197(1974).
25. S. Costa, A. Jossang, and B. Bodo. Propietes biologiques et phytochiie des Kalanchoe pinnata, Systematique ecophysiologie et phytochimie Paris: Karthala. 219-235 (1995).
26. Lieske JC, Segura JW (2004). "Ch. 7: Evaluation and Medical Management of Kidney Stones". In Potts JM (ed.). Essential Urology: A Guide to Clinical Practice (1st ed.). Totowa, New Jersey: Humana Press. pp. 117–52. ISBN 978-1-58829-109-7.
27. Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, et al. (December 2012). "Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010". Lancet. 380 (9859): 2095–128.
28. Moe OW (January 2006). "Kidney stones: pathophysiology and medical management" (PDF). Lancet. 367 (9507): 333–44.
29. Miller NL, Lingeman JE (March 2007). "Management of kidney stones." BMJ. 334 (7591): 468–72.
30. Morgan MS, Pearle MS (March 2016). "Medical management of renal stones." BMJ. 352: i52.
31. Pak CY (December 1991). "Etiology and treatment of urolithiasis." Am. J. Kidney Dis. 18 (6): 624–37.
32. Mandel NS, Mandel GS (December 1989). "Urinary tract stone disease in the United States veteran population. II. Geographical analysis of variations in composition". J. Urol. 142 (6): 1516–21.
33. Hochreiter W, Knoll T, Hess B (February 2003). "[Pathophysiology, diagnosis and conservative therapy of non-calcium kidney calculi]". Ther Umsch (in German). 60 (2): 89–97.

ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 66 Issue 06 | 2023
DOI 10.17605/OSF.IO/6U9XQ

34. Messa P, Marangella M, Paganin L, Codardini M, Cruciatti A, Turrin D, Filiberto Z, Mioni G (September 1997). "Different dietary calcium intake and relative supersaturation of calcium oxalate in the urine of patients forming renal stones." Clin. Sci. 93 (3): 257–63.

35. Bichler KH, Eipper E, Naber K, Braun V, Zimmermann R, Lahme S (June 2002). "Urinary infection stones." Int. J. Antimicrob. Agents. 19 (6): 488–98.

36. Fadeyi, M.O., A.O. Adeoye, and I.D. Olowokudejo, 1987. Epidermal and phytochemical Studies in the genus *Bohervia* (Nyctaginaceae) in Nigeria. Int. J. Crude drug research. 27: 178-184.

37. Harbone, J.B., 1998. Phytochemical methods: A guide to modern techniques of plant analysis. 3rd Edition. Chapman and Hill, London. p.279.

38. Hennequin, C., Lalanne, V., Daudon, M., Lacour, B. and Drueke, T. (1993). A new approach to studying inhibitors of calcium oxalate crystal growth. *Urol. Res.,* 21, 101-108.

39. Burns, J.R. and Finlayson, B. (1980). A proposal for standard reference artificial urine in in-vitro urolithiasis experiments. J. Urol., 18, 167-169.

40. Hess, B., Nakagawa, Y. and Coe, F.L. (1989). Inhibition of calcium oxalate monohydrate crystal aggregation by urine proteins. Am. J. Physiol., 257, F99- F106.