EVALUATION OF *IN-VITRO* ACTIVITY OF AERIAL PART OF BARANJASIF (*Achillea millefolium* Linn.) EXTRACTS

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ABSTRACT

Hasah al-Kulya (renal calculi) is one of the leading problems affecting kidney. Recurrence of kidney stone formation is approximately 50%, after 2-3 years of recovery. This makes the prevention of its recurrence an essential problem to address. Despite substantial progress in medical therapy, there is no drug with satisfactory effect. Furthermore, the drugs those are being used for its treatment have many side effects. Baranjasif (*Achillea millefolium* Linn.) is one of the drugs used in Unani Medicine that has been extensively used for the management of urolithiasis. The aim of this study is to evaluate the lithotriptic action of Baranjasif *in-vitro*. In this study, calcium oxalate stones were prepared experimentally followed by the preparation of semipermeable membrane by dissolving the outer shell of egg in 2M HCl overnight. Calcium oxalate stones weighing 10mg were then allowed to react with 7 extracts of *A. millefolium* L. viz, Pet-ether, Chloroform, Ethyl Acetate, Methanol, Acetone, Hydro-alcohol and Aqueous by packing them together in semipermeable membrane. The reaction was performed on three different concentrations of each extract i.e., 50 mg, 100 mg and 150 mg. In addition to 7 groups, two more groups were taken, i) a negative group containing only the stone of weight 10 mg and ii) standard group consisting of stone weighing 10 mg and cystone (in concentrations of 50 mg, 100 mg and 150 mg). Statistical methods like mean standard deviation, percentage dissolution, percentage inhibition, IC₅₀ and Pearson's correlation were applied to understand the extent of dissolution of stones in each group. The study advocates that Baranjasif reveals lithotriptic activity comparable to standard drug. The efficacy of the drug is directly proportional to its concentration. Ethyl acetate extract of the plant showed the most promising results.

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INTRODUCTION

Among urinary disorders, urolithiasis is the third most common prevalent disease involving the process of supersaturation, nucleation, aggregation and retention at the site of stone formation [1,2]. Its prevalence increases as age

advances. As compared to women, it is common in men sharing a ratio of 1:8 [3,4,5]. The solid accumulation of materials in kidneys results in renal stone formation whose size ranges from a grain to that of a golf ball. There are many types of renal stones. Most commonly kidney stones contain

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calcium in combination with either oxalate or phosphate constituting about 80% of kidney stones [6]. More than 60% of urolithiatic patients have calcium oxalate (CaOx) stones [7]. Struvite stones constitute nearly 15%, whereas uric acid and cysteine constitute 5-10% and 1-2% of renal stone disease respectively.

Management of urolithiasis include both medicinal as well as surgical procedures [8]. The conventional medicines to prevent hypercalciuria and hyperoxaluria induced kidney stones are thiazides diuretics and alkali citrate but scientific evidence for their effectiveness is less convincing [9]. On the other hand, removal procedures of kidney stones like surgery, Extracorporeal Shock Wave Lithotripsy (ESWL), Percutaneous Nephrostolithotomy (PCNL), etc. are expensive and as such everyone cannot afford it. Also, shock waves have traumatic effect and the residual stone fragments may persist even after the treatment which increases the chances of infection. In addition, ESWL causes hemorrhage, acute renal injury, decrease in renal function and hypertension [2]. Despite the above facts, recurrence rate of stone formation is high and follow up for a couple of years is still needed [10]. Efforts have been made to provide better alternatives of medical therapies and develop new agents which can be used either singly or in combination to prevent urolithiasis efficiently. In recent years, pronounced interest has been given towards the examination of drugs from plant products (phytotherapy) for the reasons of side effects of conventional drugs, inefficiency of synthetic conventional drugs, drug abuse etc. [11].

There are many systems of treatment available which are alternative to allopathic system (conventional medicine) such as traditional Chinese medicine, Ayurveda, Siddha, Homeopathic, Unani, etc. Unani system of medicine has holistic approach of treatment, i.e., instead of treating the symptoms, the whole body is treated. Since these drugs have relatively lesser side effects, people nowadays find herbal medicine more reliable than conventional. Therefore scientific validation of herbal medicine is the need of the hour. For the treatment of renal calculi there are a number of single drugs, e.g. *Aalubalu* (*Prunus persica*), *Duqu* (*Peucedanum grande*), *Beekh-i-Nil* (*Ipomea purga* root), *Hajrul Yahood* (*Lapis judaicus*), *Khar-i-Khasak* (*Tribulus terrestris*), *Kitmi* (*Althea officinalis*), *Nawshadar*, etc. as well as compound drugs e.g. *Banadiq-ul-Buzoor*, *Jawarish Zarooni*, *Ma'jun Aqrab*, *Ma'jun Hajrul Yahood*, etc. used in Unani system of medicine [12,13,14].

Achillea millefolium Linn. is one of the important medicinal plant which is used as lithotriptic drug in Unani system of Medicine [12,15]. *Achillea millefolium* Linn. belongs to the family Asteraceae [16] and is a widely distributed specie. Although it is native to Western Asia and Europe but is also found abundantly in most temperate regions including North

America [17]. It is also found in western Himalaya, from Kashmir to Kumaon, 6000-9000 ft above sea level [18]. In addition to *Mufattit-i-Hasāt* (lithotriptic) property, this drug has also been claimed to possess the properties of *Mudirr-i-Bawl* (diuretic), *Mufattih* (deobstruent), *Muhallil* (anti-inflammatory), *Mulattif* (demulcent), *Mudirr-i-hayd* (Emmenagogue), *Mukhrīj-i-Janīn* (Abortifacient), *Musakhkhin* (calorific), *Dafi' Hūmmā* (antipyretic) etc. [12,15,19,20,21].

Achillea millefolium Linn. has also been used for the treatment of diseases like high blood pressure, hemorrhoids, bruises, menstrual disorder, malaria, hepatitis, jaundice, headache, cough, influenza, as hepatoprotective etc. [22]. Many studies have been done to illustrate and verify that *A. millefolium* Linn. possesses pharmacological activities like anti-inflammatory, antioxidant, antifungal, antitumor, antiseptic, spasmolytic, hemostatic, cholagogue, hepatoprotective effects which are due to the various chemical constituents present in it viz., Sesquiterpene, phenolic compounds and essential oils [23,24,25,26,27,28]. The most active part of the plant is its flowering tops which contain the essential oil and are used chiefly for the treatment of hemorrhage, diarrhea, influenza, dysmenorrhea and as a haemostatic [29, 30].

MATERIALS AND METHODS

Plant Material Collection

The study was carried out at department of Ilmul Advia, Regional Research Institute of Unani Medicine (RRIUM) Srinagar, deptt. of Pharmaceutical Science and Department of Chemistry in Kashmir University. The fresh plant *Achillea millefolium* Linn. was collected in July, 2018 at an altitude of 2,740 m from Pahalgam, Anantnag, Kashmir. The plant was identified and authenticated by Dr. Akhtar H. Malik, Centre for Biodiversity and Taxonomy, University of Kashmir and submitted the specimen in KASH herbarium under Specimen Voucher no. 2700-(KASH), Department of Botany, University of Kashmir.

Evaluation for Anti-urolithiatic Activity:

Step I- Preparation of experimental kidney stones (calcium oxalate stones) by homogenous preparation:

Calcium oxalate stone was chosen to observe the dissolution, for the reason that 80% of kidney stones are calcium stones which include calcium oxalate stones and calcium phosphate stones with calcium oxalate stones constituting 60% of calcium stones. Furthermore, in industrial areas about 70% of urinary stones are calcium oxalate stones [31]. The stones used in this study were prepared in the laboratory as follows:

First of all 2N sulphuric acid (H_2SO_4) was prepared by adding 5.5ml of H_2SO_4 in 94.5 ml of distilled water. After that, 1.47 g of calcium chloride dihydrate was dissolved in 100 ml of

distilled water and 1.34 g of sodium oxalate was dissolved in 100 ml of 2N H_2SO_4 to make an equimolar solution. The so obtained equimolar solution of calcium chloride dihydrate in distilled water and sodium oxalate in 2N H_2SO_4 were allowed to react in a beaker to precipitate out calcium oxalate with stirring. Precipitates were freed from traces of H_2SO_4 by ammonia solution. The precipitates were washed with distilled water and dried at $60^\circ C$ for 4 hours [32]. **Fig. 1 (a)** shows the photograph of experimentally prepared calcium oxalate stones used in this study.

Fig. 01: Photograph showing (a) experimentally formed calcium oxalate stones, (b) experimentally formed egg semipermeable membrane, (c) Sutured egg semipermeable membrane, (d) Stone and extracts in tris buffer (e) Titration Process

Step II- Preparation of semi-permeable membrane from farm eggs:

The semi-permeable membrane of egg lies in between the outer calcified shell and the inner contents viz: albumin and

yolk. Apexes of eggs were punctured by a glass rod in order to squeeze out the entire content. Empty eggs were washed thoroughly with distilled water and placed in a beaker containing 2 M Hydrochloric acid (HCl) for overnight, causing complete decalcification. Subsequently, the content was washed with distilled water, placed in ammonia solution for neutralization of acid traces in the moistened condition for a while and rinsed with distilled water and stored in refrigerator at a pH of 7-7.4 [32]. **Fig. 1 (b)** shows experimentally formed egg semi-permeable membrane.

Fig. 2. Graphical representation of dissolution of calcium oxalate stone by various extracts of *A. millefolium* Linn

Step III- Estimation of calcium oxalate by Titrimetry:

The procedure for estimation of calcium oxalate in this study has been adopted from Jha, *et al.*, 2016 and Saravana Singh *et al*, 2016 with slight modifications [33,34]. The dissolution percentage of calcium oxalate was evaluated by taking exactly 10 mg of calcium oxalate and 50, 100, 150 mg of the extracts packed together in the semipermeable membrane of egg by suturing. The contents were allowed to suspend in a conical flask containing 100 ml of 0.1M Tris buffer (0.1M of tris buffer was prepared by adding 12.1gm of tris buffer in 1000 ml of distilled water). Several groups of extracts corresponding to different concentrations of drugs were formed as shown in **table 1**. In addition to that two more groups, one serving as blank, containing only 10 mg of calcium oxalate and the other as positive control, containing 10 mg of calcium oxalate mixed with different concentrations of standard drug Cystone were formed. The incubator was preheated at $37^\circ C$ for 2 hours and all the groups were placed in the incubator at $37^\circ C$ for 7-8 hours. The contents of each semi-permeable membrane were removed into test tubes. 2 ml of 1N H_2SO_4 was then added in each test tube. The contents of test tubes were then titrated with 0.01N Potassium permanganate ($KMnO_4$) till a light pink color (end point) was obtained. 1ml of 0.01N $KMnO_4$ is equivalent to 1.25 mg of calcium oxalate. **Fig 01. (c)** shows the photographs of sutured egg semi-permeable membrane, **(d)** beakers containing extracts of *Achillea millefolium* Linn. along with calcium oxalate stones and **(e)** titration process.

Table 1: Percentage of dissolution of calcium oxalate stone (10 mg) by various extracts of *Achillea millefolium* Linn.

S. No.	Group	Concentration of drug (in mg)	Vol. of standard KMnO ₄	Wt. of Calcium estimated	Wt. of Calcium reduced	Percentage of Dissolution	Percentage of Inhibition
1.	Group I (Negative control)	-	8ml	10mg	-	-	100%
2.	Group II (Standard, containing cystone)	50	5.9ml	7.375	2.625	26.25%	73.75%
		100	5.6ml	7.00	3.00	30%	70%
		150	4.9ml	6.125	3.875	38.75%	61.25%
3.	Group III (containing Pet-ether extract)	50	8ml	10	-	-	100%
4.	Group IV (containing Chloroform extract)	100	8ml	10	-	-	100%
		150	8ml	10	-	-	100%
		50	8ml	10	-	-	100%
		100	8ml	10	-	-	100%
		150	8ml	10	-	-	100%
5	Group V (containing Ethyl-acetate extract)	50	6.6ml	8.25	1.75	17.5%	82.5%
		100	5.8ml	7.25	2.75	27.5%	72.5%
		150	5.5ml	6.875	3.125	31.25%	68.75%
6	Group VI (containing Methanol extract)	50	7ml	8.75	1.25	12.5%	87.5%
		100	6.5ml	8.125	1.875	18.75%	81.25%
		150	6.2ml	7.75	2.25	22.5%	77.5%
7	Group VII (containing Acetone extract)	50	7.1ml	8.875	1.125	11.25%	88.75%
		100	6.5ml	8.125	1.875	18.75%	81.25%
		150	6.4ml	8.00	2.00	20%	80%
8	Group VIII (containing Hydro-alcohol extract)	50	7.5ml	9.375	0.625	6.25%	93.75%
		100	6.8ml	8.50	1.5	15%	85%
		150	6.6ml	8.25	1.75	17.5%	82.5%
9	Group IX (containing Aqueous extract)	50	6.3ml	7.875	2.125	21.25%	78.75%
		100	6ml	7.5	2.5	25%	75%
		150	5.8ml	7.25	2.75	27.5%	72.5%

To know the quantity of calcium oxalate the test substance could dissolve, the amount of undissolved calcium oxalate was subtracted from the total quantity used in the experiment in the beginning.

The percentage dissolution of calcium oxalate stone (10 mg) in various extracts of *Achillea millefolium* Linn was

observed. The statistical analysis was carried out by calculating percentage inhibition, mean standard deviation of various trials, half maximal inhibitory concentration (IC₅₀), and Pearson's correlation among the extracts.

RESULTS AND DISCUSSIONS

Kidney stones occurs due to supersaturation of urine with the

Table 2: Descriptive statistics for individual extracts with concentrations.

	Concentration of drug (mg)	N	Mean%±SD	Min	Max	IC ₅₀
Group II (Standard containing cystone)	50	3	26.25±0.31	26.1	26.75	237
	100	3	30±2.3	27.33	31.22	
	150	3	38.75±1.9	37.55	39.33	
Group III (Pet-ether extract)	50	3	-	-	-	
	100	3	-	-	-	
	150	3	-	-	-	
Group IV (Containing Choloform extract)	50	3	-	-	-	
	100	3	-	-	-	
	150	3	-	-	-	
Group V (Containing Ethyl-acetate extract)	50	3	17.5±0.8	16.15	18.33	274
	100	3	27.51±1.7	25.31	29.3	
	150	3	31.25±1.3	28.3	32.6	
Group VI (Containing Methnol extract)	50	3	12.5±0.8	11.05	13.33	421
	100	3	18.75±1.7	16.11	20.31	
	150	3	22.50±1.3	21.31	23.6	
Group VII (Containing Acetone extract)	50	3	11.25±2.3	9.05	12.33	467
	100	3	18.75±0.9	17.11	21.31	
	150	3	20±0.10	19.31	23.65	
Group VIII (Containing hydro Alcohol extract)	50	3	6.25±3.3	4.05	10.33	439
	100	3	15±1.9	13.11	18.01	
	150	3	17.5±0.80	16.21	18.65	
Group IX (Aqueous)	50	3	21.25±2.3	19.05	22.32	528
	100	3	25±2.9	23.33	26.65	
	150	3	27.5±0.10	26.21	28.01	

substances occurring in it as a result of which these substances crystallize to form stones. There are many factors associated with the occurrence of renal calculi like dietary habits, climate geographical area, age, gender, drugs etc. [35].

In classical text books of Unani System of Medicine, management of urinary stone has been discussed by many renowned Unani physicians in a systematic way. For treatment of renal calculi, they recommended 1) *Ilaj Bil-Ghiza* (dietotherapy); 2) *Ilaj Bit-Tadbeer* (regimental therapy) 3); *Ilaj Bid-Dawa* (pharmacotherapy) and 4) *Ilaj bilYad* (surgery). In Dietotherapy i.e. *Ilaj Bil-Ghiza*, foods which are soft, easy to digest and produce good chyme are allowed to eat. In addition, plenty of fluid is advised. Foods containing high content of oxalates, hard to digest and those

which produce morbid matters, are avoided. *Ilaj Bit-Tadbeer* (regimental therapy) has two main aims, *Talteef-i-Madda* (softening of morbid matter) and *Taqti'-i-Madda* (refining) for which patient is given emetic or purgatives drugs or diuretics. *Fasd* (Venesection) can also be done on Basilic vein (*Rag-i-Basaleeq*). Other regimental therapies include *Huqna* (Enema), *Abzan* (Sitz bath) etc. *Ilaj Bid-Dawa* (pharmacotherapy) follows the principle to breakdown the stone and its expulsion which is illustrated as *Tafteet-i-Hasat* and *Idrar-i-Bawl* respectively. If the patient is not relieved by the above line of treatment then, this is followed by surgical procedures [36,37,12].

In classical text books of Unani Medicine, it has been mentioned that lithotriptic drugs must possess mild degree of heat as severe degree of heat makes the substance harder. Ibn-

Table 3: Correlation between extract and concentrations used in the study.

		Correlations					
		Cystone	Ethyl-Acetate Extract	Methnol Extrac	Acetone Extract	Alcohol Extract	Aqueous
Cystone	Pearson Correlation	1	.852**	.924**	.804**	.795*	.875**
	Sig. (2-tailed)		.004	.000	.009	.010	.002
	N	9	9	9	9	9	9
Ethyl-Acetate Extract	Pearson Correlation	.852**	1	.982**	.984**	.968**	.969**
	Sig. (2-tailed)	.004	.000	.000	.000	.000	
	N	9	9	9	9	9	9
Methnol Extract	Pearson Correlation	.924**	.982**	1	.965**	.958**	.984**
	Sig. (2-tailed)	.000	.000		.000	.000	.000
	N	9	9	9	9	9	9
Acetone Extract	Pearson Correlation	.804**	.984**	.965**	1	.978**	.969**
	Sig. (2-tailed)	.009	.000	.000		.000	.000
	N	9	9	9	9	9	9
Alcohol Extract	Pearson Correlation	.795*	.968**	.958**	.978**	1	.980**
	Sig. (2-tailed)	.010	.000	.000	.000		.000
	N	9	9	9	9	9	9
Aqueous	Pearson Correlation	.875**	.969**	.984**	.969**	.980**	1
	Sig. (2-tailed)	.002	.000	.000	.000	.000	
	N	9	9	9	9	9	9
**. Correlation is significant at the 0.01 level (2-tailed).							
*. Correlation is significant at the 0.05 level (2-tailed).							

Sina has mentioned that for the treatment of renal calculi, compound drug must possess both lithotriptic and diuretic property. The reason behind it is that lithotriptic action of drug breaks down the stone while its diuretic action causes excessive diuresis and helps the fragments to pass out through urine [12].

Achillea millefolium Linn. is one among those drugs possessing temperament of mild heat. It possesses property of lithotriptic and diuretic action which fulfills the quality of lithotriptic drugs as said by Ibn-Sina. It has been used by Unani physicians since a long period. It is used alone or in

combination with other drugs either orally, or locally as sitz bath, enema etc. Baranjasif also possesses anti-microbial characteristics which may play an additional role in prevention of formation of secondary stones caused by infections in urine. Many scientific studies have been carried out on this plant e.g. antimicrobial activity, estrogenic activity, choleric activity, antioxidant activity etc. But its lithotriptic action has not been proven scientifically yet. Therefore, this study was taken up to provide experimental evidence for the claim made by Unani physicians about the efficacy of *Achillea millefolium* Linn. in the treatment of urolithiasis.

Fig. 3: Mean percent dissolution of calcium oxalate stone in: (a) cystone, (b) ethyl-acetate extract, (c) Methanol extract, (d) Acetone extract, (e) hydro alcohol extract and (f) Aqueous extract.

Various extracts of aerial part of the plant was studied for its lithotriptic activity.

The percentage dissolution of calcium oxalate stone (10 mg) in various extracts of *Achillea millefolium* Linn is shown in table 1 and fig. 2 and 3. The statistical analysis was carried out by calculating percentage inhibition as given in table 1, mean standard deviation of various trials, half maximal inhibitory concentration (IC_{50}), given in table 2 and Pearson's correlation among the extracts (table 3).

The standard group showed significant dissolution rate of 26.25%, 30% and 38.75% at the concentrations of 50 mg, 100 mg and 150 mg respectively. Ethyl acetate extract of *Achillea millefolium* Linn. on the other hand, showed comparable results with dissolution rates of 17.5%, 27.50% and 31.25 at the concentrations of 50 mg, 100 mg and 150 mg respectively. The dissolution rates of aqueous extract were 21.25%, 25%, 27.5 % at the concentrations of 50 mg, 100 mg and 150 mg respectively. The dissolution rates of methanol extract were 12.5%, 18.75%, 22.5% at the corresponding concentrations respectively. Aqueous extract was found to possess better dissolution rate than the methanol extract of the plant. Acetone extract showed lesser rates of dissolution

comparatively with the dissolution rates of 11.25%, 18.75% and 20% and the least activity was found in Hydro-alcohol extract with the dissolution rates of 6.25%, 15 and 17.5% at the corresponding concentrations respectively. No activity was seen in pet-ether and chloroform extract implying the inefficiency of these groups in treatment of the disease caused due to the calcium oxalate kidney stones. This indicates that the mean percentage dissolution of calcium oxalate is maximum in standard drug as compared to the rest of the extracts of *Achillea millefolium* Linn. However, ethyl acetate extract of the plant also shows the comparable degree of dissolution.

Furthermore, it is evident from the figure 4 that the mean percentage dissolution of calcium oxalate tends to increase with increase in the concentration of extracts. In order to get a clearer picture, the percentage inhibition was plotted against the concentrations of Cystone and various extracts of *Achillea millefolium* Linn (figure 5). IC_{50} for each case were estimated by following the linear regression. The values of IC_{50} , given in table 2, clearly indicate that the response of ethyl acetate extract of the plant is similar that of Cystone. In addition, there is a positive correlation between extracts and

Fig. 4: Percentage inhibition of cystine and various extracts of *A. millefolium* Linn.

the concentration used in the given study. More specifically, it means that if the concentration of extract increases percentage dissolution of calcium oxalate increases up to some limit, and hence the efficacy increases. Pertinent to mention here that the concentration is highly significant if p -value < 0.01 and significant if p -value is < 0.05 .

CONCLUSION

On the basis of the above findings it is concluded that, *Achillea millefolium* Linn. possesses lithotriptic action which increases with the increase in concentration of drug implying that the efficacy of the drug is directly proportional to the concentration of drug. This study serves the base for validation of the lithotriptic action of Baranjasif (*Achillea millefolium* Linn.). The Unani concept that the lithotriptic drug acts by means of *Mufattit*, *Muhallil* and *Mufattih* *Sudad* property of Baranjasif is also validated in this study. As this study is confined to in-vitro case, so both in-vivo and clinical trials should be carried out in future to further examine the lithotriptic activity of Baranjasif (*Achillea millefolium* Linn.) in a broader perspective. A rigorous study in future may include several trials with varying concentrations of extracts to carry out the extensive regression analysis to reach the precise results.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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