EVALUATION OF ANTINEPHROLITHIATIC POTENTIAL OF SOME ETHNOMEDICINAL PLANTS OF UTTARAKHAND, INDIA

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Abstract:

Objective: *The aim of this study was to investigate the effect of ten ethnomedicinal plants used by local healers of Himalayan region of Uttarakhand state of India on invitro inhibition of calcium oxalate stones crystallization and its dissolution.*

Material and method: *Different parts of the plants were used to make hexane, methanol and aqueous extracts. In vitro anti-nephrolithiatic activity was tested in terms of the inhibitory activity of the plant extracts on the nucleation, aggregation and crystal growth of calcium oxalate crystals in presence and absence of plant extracts using spectrophotometric methods. The effect of plant extract on in vitro dissolution of prepared CaoX crystals was done with the help of Titrimety method and percentage dissolution was compared to standard drug cystone.*

Result: *All ethnomedicinal plants showed varied degree of calcium stone inhibition and dissolution. Highest mean anti nephrolithiasis activity was shown by aqueous extract of Macrotyloma uniflorum (91.12 %) followed by Cedrus deodara (90 %). In vitro dissolution of CaoX crystal showed that all plants in all three solvents showed varied degree of dissolution as compared to standard cystone. The highest dissolution percentage of oxalate calcium stones was obtained for the aqueous extract of Macrotyloma uniflorum (78.39 ± 1.10).*

Conclusion: *All ethnomedicinal plants showed varied degree of calcium stone inhibition and dissolution with Macrotyloma uniflorum displaying most promising results. These findings substantiate the traditional use of the plants in the treatment of urinary stones and kidney problems. The outcome of the present investigation will be helpful for formulation of anti-nephrolithiatic drug after in vivo studies on experimental animals.*

Key Words: *Anti-nephrolithiatic, Calcium oxalate stones, Nephrolithiasis, ethnomedicinal plants.*

1. INTRODUCTION:

Nephrolithiasis or kidney stone formation is a condition marked by its recurrence: the recurrence rate of stone is approximately 10% within one year, 35% within five years, and 50% within 10 years [1]. Calcium oxalate type of calculi is most common of all types, which are formed from oxalates in urine made by the body. About 75% of kidney stones are composed of oxalate crystals which is enhanced by certain special types of food which includes food with high level of oxalates (Spinach, nuts etc.) where it combines with calcium to form calcium oxalate stones. Although many advanced surgical procedures are there for removal of these stone but the cost associated with it and its recurrence impose a major limitation to such advancement [2]. One of the best ways to prevent and treat urolithiasis (anti-lithiasis) is to control the process of crystallization events which includes nucleation, aggregation, crystal growth [3]. This is best achieved by the use of herbal extracts since they have been widely used in folk medicine to treat kidney stones. Herbal remedies, are regarded as quite safe with minimal or no side effects, cost effective, readily available and easily affordable. Several plant extracts have been used to treat kidney stones with promising effect in prevention and treatment [2]. Some of these are used as dietary plants.

The present study was carried out for the assessment of some of ethnomedicinal plants of Uttarakhand state of India for *in-vitro* crystallization and dissolution assay.

2. Materials and Methods:

2.1 Chemicals

High grade chemicals were purchased from Himedia Pvt Limited (LR).

2.2 Plant material

The plant material listed as listed in table 1 and shown in fig.1 were collected from nearby areas of GBPIET Pauri Garwhal. The plants were identified with the help of indigenous knowledge of local people and further verified in BSI (Botanical survey of India) Dehradun Uttarakhand.

2.3 Method

2.3.1 Preparation of plant extracts: The above-mentioned plant materials were made completely clean, dust free, shade dried and the powdered material were successively prepared. Plant extracts were prepared using Soxhlet extraction method using hexane, methanol and water as solvents. All the extracts were stored in tightly closed glass bottles in refrigerator at 2-8ºC.

2.3.2 Primary phytochemical screening

Preliminary phytochemical screening was carried out for the presence of secondary metabolites; such as saponin, phenol, carbohydrate, amino acids, flavonoids, Tannins using methods given by Banu and Catherine (2015) [4].

2.3.3 Evaluation of plant extracts for prevention of kidney stone

The study was augmented over three different assays viz: nucleation. aggregation and crystal growth assay for studying in-vitro crystallization of calcium oxalate stones.

2.3.4 Evaluation of *in-vitro* anti-nephrolithiatic activity by Turbidity Method

In vitro anti-nephrolithiatic activity was tested in terms of the inhibitory activity of the plant extracts on the nucleation, aggregation and crystal growth of calcium oxalate crystals in presence and absence of plant extracts.

Nucleation assay: Effect of different plant extract on calcium oxalate (CaOx) crystal formation was determined by means of nucleation assay. Calcium chloride ($CaCl_2$) (5 mmol L^{-1}) and sodium oxalate ($Na_2C_2O_4$) solution (7.5 mmol L^{-1}) were prepared in Tris-HCl (0.05 mol L^{-1}) and NaCl (0.15 mol L^{-1}) buffer (pH 6.5). One milliliter of each plant extract was mixed with 3 ml $CaCl_2$ solution followed by the addition of 3 ml $Na_2C_2O_4$ solution. Final mixtures were incubated for 30 min at 37ºC. The optical density (OD) of the mixtures was then measured at 620 nm wavelength. Percent inhibition of nucleation by plant extracts was calculated using the under mentioned formula:

% Inhibition of nucleation = [(C-S) /C] ***** 100 ……. Equation (1)

Where, C is the turbidity without extract. S is the turbidity with extract [5].

Aggregation assay: Effect of different plant extracts on CaOx crystal aggregation was determined by means of aggregation assay. $CaCl_2$ and $Na_2C_2O_4$ solutions (50 mmol L^{-1} each) were mixed together, heated to 60 ºC in a water bath for 1 hour. It was incubated overnight at 37ºC to prepare seed CaOx crystals. After drying, CaOx crystal solution (0.8 mg ml^{-1}) was prepared in a 0.05 molL^{-1} Tris-HCl and 0.15 mol L^{-1} NaCl buffer (pH 6.5). One millilitre of each plant extract was added to 3 ml CaOx solution, vortexed and then incubated at 37ºC for 30 min. OD of the final mixtures was then read at 620 nm wavelength and percent inhibition of aggregation was then calculated as described for nucleation assay in equation (1) [5].

Crystal growth assay: Briefly, 20 ml each of 4Mm calcium chloride and 4Mm of sodium oxalate were added to a 30ml of solution, containing NaCl (90 mM) buffered with tris HCl (10mM) pH 7.2. To this 600 µl of calcium oxalate monohydrate (COM) crystal slurry as well as to this 1.5 mg ml^{-1} acetate buffer was added. Consumption of oxalate begins immediately after COM slurry addition and was monitored for 30 minutes for disappearance of absorbance at 214nm.1 ml of each plant extract was separately added into reaction mixture. The depletion of free oxalate ions will decrease if extract inhibits calcium oxalate crystal growth. Rate of reduction of free oxalate was calculated using the baseline value and the value with or without extract after 30 min of incubation. The percentage inhibitory activity was calculated by Formula:

% Inhibitory activity = [(C-S)/C] * 100……. Equation (1)

Where, C is the rate reduction of free oxalate without extract. S is the rate reduction of free oxalate with extract [6].

a. Preparation of laboratory calcium oxalate stone

Equimolar solution of calcium chloride dihydrate was dissolved in distilled water and sodium oxalate in 10ml of $2N\ H_2SO_4$ was allowed to react in sufficient quantity of distilled water in a beaker. The resulting precipitate was calcium oxalate. It was washed with distilled water and dried at 60˚C for 4 hours.

2.4 Preparation of semi permeable membrane from farm egg

The semi permeable membrane of eggs lies in between the outer calcified shell and the inner contents like albumin and yolk. The shell was removed chemically by placing the eggs in 2M HCL for an overnight which caused complete decalcification and washed with distilled water. Carefully with sharp pointer a hole was made on the top and the contents squeezed out completely from the decalcified egg. The membrane was rinsed with distilled water and stored in refrigerator.

2.5 Estimation of percentage dissolution of CaoX stones.

The dissolution percentage of calcium oxalate was calculated by taking exactly 1 mg of calcium oxalate and 10mg of different plant extracts, 10 mg of Cystone (standard) packed it together with egg semipermeable membrane. Cystone is a marketed composite herbal formulation specifically developed for managing urolithiasis or renal calculi [7]. This was allowed to suspend in a conical flask containing 100ml of 0.1M Tris buffer. 1 mg of calcium oxalate in egg semipermeable membrane acts as the control. All the conical flask containing semipermeable membrane were kept in an incubator at 37ºC for 2hours. Remove the contents of semipermeable membrane in to separate test tube, add 2ml of 1N sulphuric acid to each test tube and titrated with 0.9494N KMnO4 till a light pink colour end point was obtained .The amount of remaining undissolved calcium oxalate is subtracted from the total quantity used in the experiment in the beginning to know the total quantity of dissolved calcium oxalate by various extracts. Each ml of 0.9494 N KMnO4 equivalents to 0.1898mg of Calcium oxalate.

The percentage dissolution was calculated as follows: Dissolved calcium oxalate = (Undissolved calcium oxalate) – (Total quantity used in the Experiment in the beginning)
Percentage dissolution=Dissolved calcium oxalate X 100……Equation…2

2.6 Statistical analysis

Quantitative results of all the experiments performed in triplicates were expressed as mean ± S.E.M. (Standard Error of Mean). Statistical computations were performed on Origin Pro version 8.5 software using one-way analysis of variance (ANOVA). Entire results were calculated by using student's unpaired t-test, wherein a p-value of less than 0.005 was reflected significant (mean *$p<0.05$.

3. Results:

3.1 Phytochemical screening

Qualitative phytochemical estimation of all ten ethnomedicinal plants in different solvent as shown in Table 2, Table 3, Table 4 revealed the presence of flavonoids, phenolic compounds, saponins, tannins, carbohydrates, amino acid.

3.2 Nucleation assay

One-way annova analysis showed that the percentage inhibition between the different solvent and within the same solvents were significantly different at $p<0.05$. From the results on in-vitro study as shown in fig.1 it was observed that with all the plant extract the nucleation was found to be inhibited and the crystal formation was prevented as compared to control without plant extract (0.82 %). Methanolic and aqueous extracts of seeds of *Macrotyloma uniflorum* showed maximum nucleation inhibition of (97.88±1.24%) and (97.11±1.7%) respectively. Second highest nucleation inhibition was show by methanolic extract of *Urtica dioica* of (90.75±0.35%). In case of hexane extract *Cynodon dactylon* showed maximum nucleation inhibition value of (72.7±1.4%). It is also clear that among all the extract for nucleation inhibition aqueous extract of all plants except *Urtica dioica* showed comparatively higher percentage of nucleation inhibition as compared to other two solvents.

3.3 Aggregation Assay

One-way annova analysis showed that the percentage inhibition between the different solvent and within the same solvents were significantly different at $p<0.05$. From the results on in-vitro study as shown in fig. 2 it was observed that with all the plant extract analysed the aggregation was found to be inhibited and the crystal formation was prevented as compared to control without plant extract (0.82 %). *Cedrus deodara* aqueous extract showed maximum aggregation inhibition of (92.64±1.61%) followed by aqueous extract of *Pyracantha crenulata (*90.96 ± 0.79 %) and methanol extract of *Cynodon dactylon* (86.4±0.71%) and *Urtica dioica (*86.4±0.11%). As in case of nucleation assay among all

the extract for aggregation inhibition aqueous extract of most plants showed comparatively higher percentage of inhibition as compared to other two solvents.

3.4 Growth assay

One-way annova analysis showed that the percentage inhibition between the different solvent and within the same solvents were significantly different at $p<0.05$. From the results on in-vitro study as shown fig. 3 it was observed that with all the plant extract analysed the crystal growth was found to be inhibited and the crystal formation was prevented as compared to control without plant extract (0.20 %). Hexane extract of *Urtica dioica* showed maximum inhibition of (98.25±0.35%) followed by aqueous extract of *Macrotyloma uniflorum* showing (92.1±0.56 %) and aqueous extract of *Cedrus deodara* showing *(*91± 1.00 %).

Hence, taking into consideration various steps in stone formation with focus on the overall consequence of stone development, the anti-nephrolithiasis activity of various plant has been calculated by fetching up the mean effect of extract on nucleation, growth and aggregation as shown in (Fig:4,5,6). From the graph it can be seen that highest mean anti-nephrolithiasis activity in case of aqueous extract is shown by *Macrotyloma uniflorum (*91.12) % followed by *Cedrus deodara (*90 %). In hexane extract highest mean anti nephrolithiasis activity shown by *Cynodon dactylon* (80.61%) followed by *Cedrus deodara* (74.61%) and *Adiantum capillus* (73.09%). Highest mean anti nephrolithiasis activity in methanol extract was shown by *Urtica dioica* (72.08%) followed by *Macrotyloma uniflorum* (62. 48%.) and *Pyracantha crenulata* (61.47 %).

3.5 Dissolution study

According to the results Annova the dissolution percentages of oxalate calcium by different extracts were significantly different at $p<0.05$. The highest dissolution percentage of oxalate calcium stones was obtained for the aqueous extract of *Macrotyloma unifloum* (78.39 ± 1.10) followed by methanol extract of same plant (75.3 ± 0.89 as shown in fig 7. Aqueous extract and methanolic extract of *Urtica dioica* also showed promising result comparable to that of *Macrotyloma uniflorum* (73.5 ± 1.89) and (72.2 ± 0.91) respectively.

4. DISCUSSION:

Phytochemicals like flavonoids, triterpenes, saponins, tannins, alkaloids, glycoside derivatives, proteins, tannins and steroids are reported to be responsible for anti-urolithiatic effect by either inhibiting the formation of calcium oxalate crystals, preventing their attachment to renal cells or by their calcium channel blocking activity [8]. Saponins possess anti-nephrolithiatic properties and are known to disintegrate mucoproteins that are crucial components of stone matrix. Tannins and polyphenols inhibit CaOx crystal formation as well as dissolve the pre-formed CaOx crystals by aiding calcium complexation [5].

Nucleation is the establishment of the smallest unit of crystal formation in a solution step in renal stone formation. Addition of $Na_2C_2O_4$ solution to the reaction mixture consisting of $CaCl_2$ resulted in the formation of numerous CaOx crystals. Nucleation basically marks a thermodynamically driven event of phase change wherein dissolved substances in a supersaturated solution spontaneously crystallize (Agarwal et al., 2013). Methanolic and aqueous extracts of seeds of *Macrotyloma uniflorum* showed maximum nucleation inhibition of (97.88±1.24%) and (97.11±1.7%) respectively. Studies done by Sharma et al.2019 [9] and Chaitanya et al., 2010 [10] have also shown the potential of aqueous and methanolic extract of *Macrotyloma uniflorum* against kidney stone.

Aggregation constitutes the most effective mechanism to increase the size of particles, composition and structure of urinary stone. Aggregation is a key determinant of crystal retention as large crystal agglomerates are the ones that produce renal tubular obstruction thereby promoting stone formation [11]. *Cedrus deodara* aqueous extract showed maximum aggregation inhibition of (92.64±1.61%). Ramesh C. et al 2010 [12] also confirmed the beneficiary effect of *Cedrus deodara* in urolithiasis.

Newly formed crystals may combine or grow to form a small, hard mass called stones. From the present study it can be seen that among 10 plants hexane extract of *Urtica dioica* can be effective for managing kidney stone formation at crystal growth assay. Among the three solvents hexane extract showed least dissolution percentage in almost all plant extract that could be attributed to absence of most of the phytochemicals or their low amount.

5. Conclusion:

Anti-nephrolithiatic potential of all the ten plant parts were investigated in different solvents with the help of nucleation, aggregation and crystal growth inhibition assays as well as dissolution assay. All the extracts showed significant anti-nephrolithiasis activity in all assays but results suggest the greater potential by aqueous and methanol extracts of *Macrotyloma uniflorum (*97.88±1.24% and 97.11 ± 1.7%) for nucleation assay, for aggregation study aqueous extract of *Cedrus deodara (*92.64±1.61%), for crystal growth inhibition hexane extract of *Urtica dioica (*98.25±0.35%). In terms of mean anti-nephrolithiasis activity highest inhibition shown by aqueous extract of *Macrotyloma uniflorum (*91.12) % followed by *Cedrus deodara (*90 %). The highest dissolution percentage of oxalate calcium stones was

obtained for the aqueous extract of *Macrotyloma unifloum* (78.39 ± 1.10). Our preliminary phytochemical investigation showed the presence of Phenols, flavonoids, tannins and saponins. The presence of these metabolites indicates pharmacological activity of plants extracts. Since the current focus of various pharmaceutical industries is on developing plant based, therapeutic drugs. Identification of mechanism of active ingredients responsible for stone inhibition and dissolution together with animal testing and critical clinical trials are required in further research and also investigations to validate the efficacy and safety of these constituents in patients with kidney stones

6. Acknowledgement

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7. Conflict of interest

There is no conflict of interest among authors.

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Table 1 Plants and their parts used

S.No	Name of plant	Part used
1	***Macrotyloma uniflorum***	Seeds
2	***Asapragous racemousa***	Roots
3	***Urtica dioica***	Leaves
4	***Cedrus deodara***	Bark
5	***Cynodon dactylon***	Whole Plant
6	***Pyracantha crenulata***	Leaves
7	***Adiantum Capillus***	Leaves

8	***Oxalis corniculata***	Leaves
9	***Duchesnea indica***	Fruits
10	***Ageratum conyzoides***	Whole plant

Table 2 Preliminary phytochemical analysis of aqueous plant extract

Phyto-constituents	*C. dactylon*	*C. deodara*	*A. racemousa*	*U. dioica*	*M. unifloru m*	*P. crenulat a*	*A. Capillus*	*O. corniculata*	*A. conyzoide*	*D. indica*
Saponin	+	++	++	+	+	+	+	-	+	-
Phenol	+	+	+	++	+	+	+	+	+	+
Carbohydrates	+	+	+	+	+	+	+	+	+	+
Aminoacids	+	++	-	+	++	+	+	+	++	+
Flavonids	++	++	++	++	++	+	+	+	+	+
Tannins	-	+	++	-	+	+	+	+	+	-

Table 3 Preliminary phytochemical analysis of methanol plant extract

Phytoconstituents	*C. dactylon*	*C. deodara*	*A. racemousa*	*U. dioica*	*M. unifloru m*	*P. crenulata*	*A. capillus*	*O. corniculat a*	*A. conyzoi de*	*D. indica*
Saponin	++	+	+	+	++	+	+	-	+	+
Phenol	+	-	-	+	-	+	+	+	+	+
Carbohydrates	+	+	+	+	+	+	+	+	+	+
Aminoacids	+	-	-	+	+	+	+	+	+	+
Flavonids	+	+	-	+	+	+	+	+	-	-
Tannins	-	-	+	-	+	+	+	+	+	+

Table 4 Preliminary phytochemical analysis of Hexane plant extract

Phytoconstituents	*C. dactylon*	*C. deodar a*	*A. racemous a*	*U. dioica*	*M. unifloru m*	*P. crenulat a*	*A. capillu s*	*O. cornicula ta*	*A. conyzoid e*	*D. indic a*
Saponin	+	++	++	+	-	-	+	+	-	-
Phenol	+	+	+	++	-	-	-	-	-	-
Carbohydrates	+	+	+	+	+	-	-	+	+	+
Aminoacids	+	++	-	+	+	-	-	-	+	-
Flavonids	++	++	++	++	+	-	-	-	+	-
Tannins	-	+	++	-	-	-	+	+	-	-

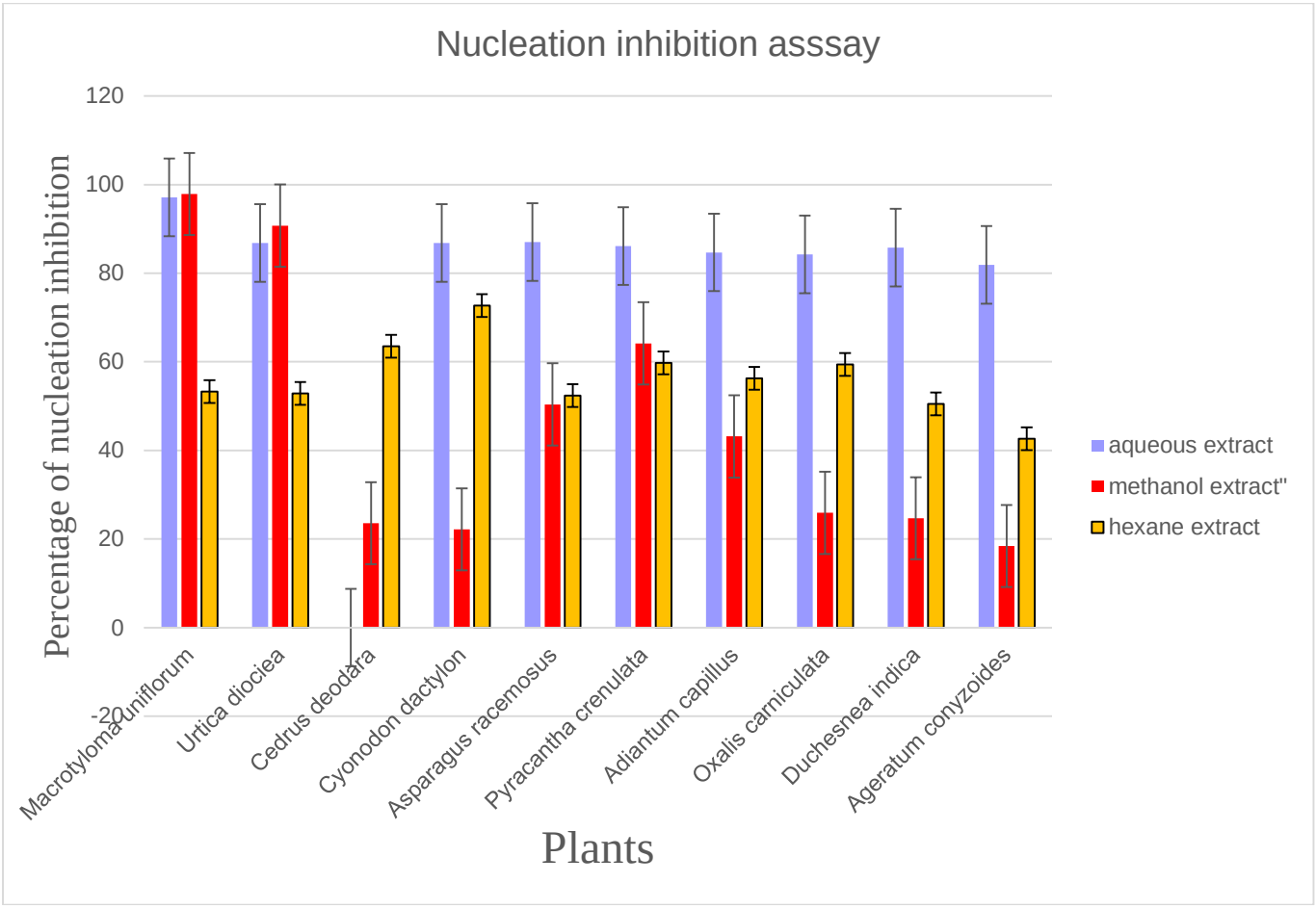

Fig 1: Nucleation inhibition of different plant extract

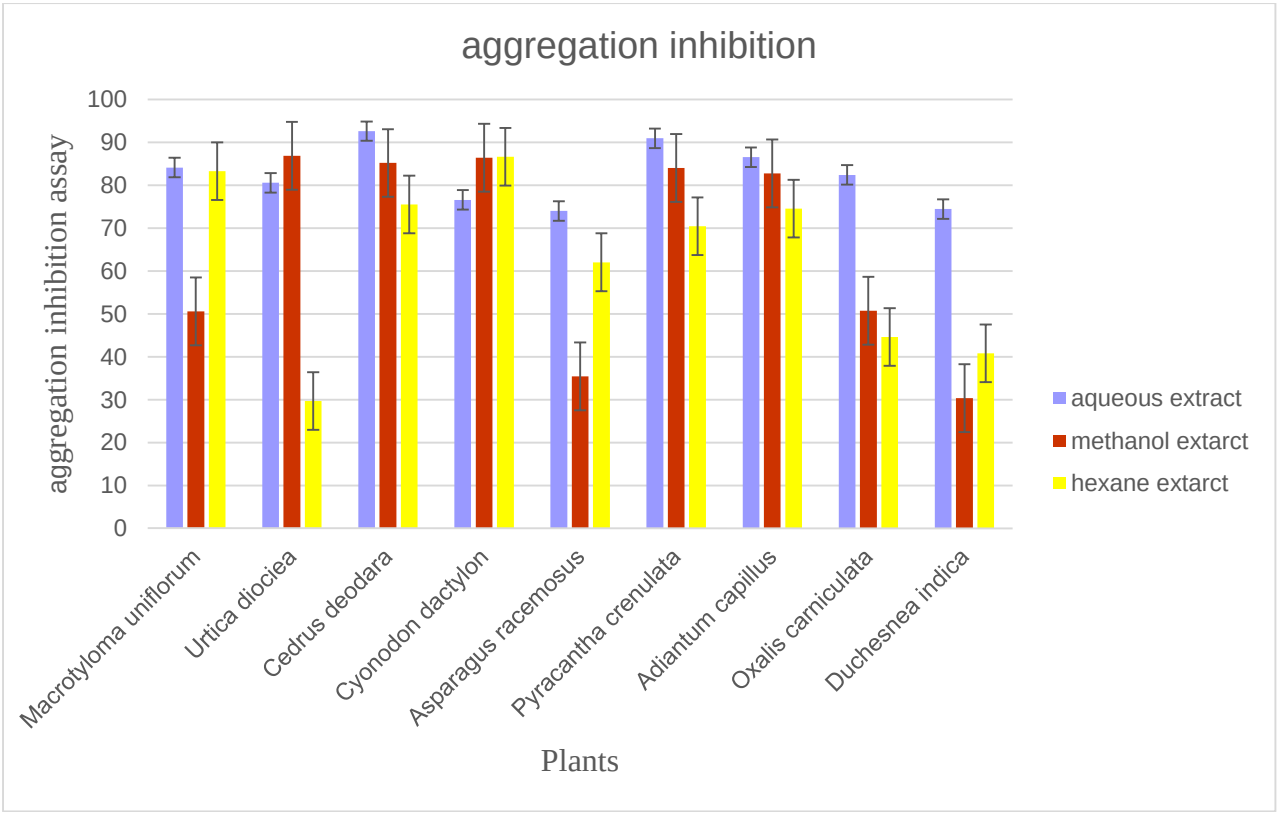

Fig 2: Aggregation inhibition of different plant extract

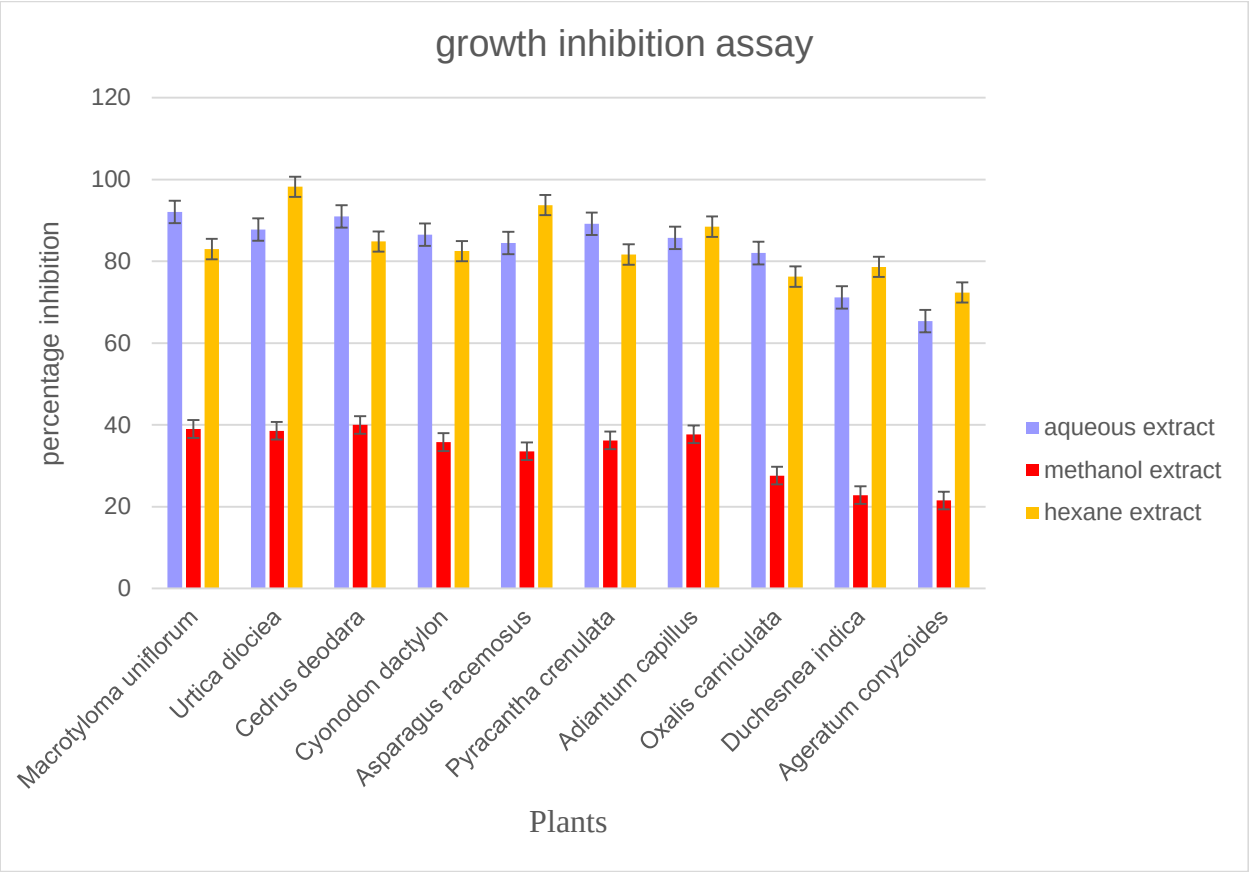

Fig 3: Growth inhibition of different plant extract

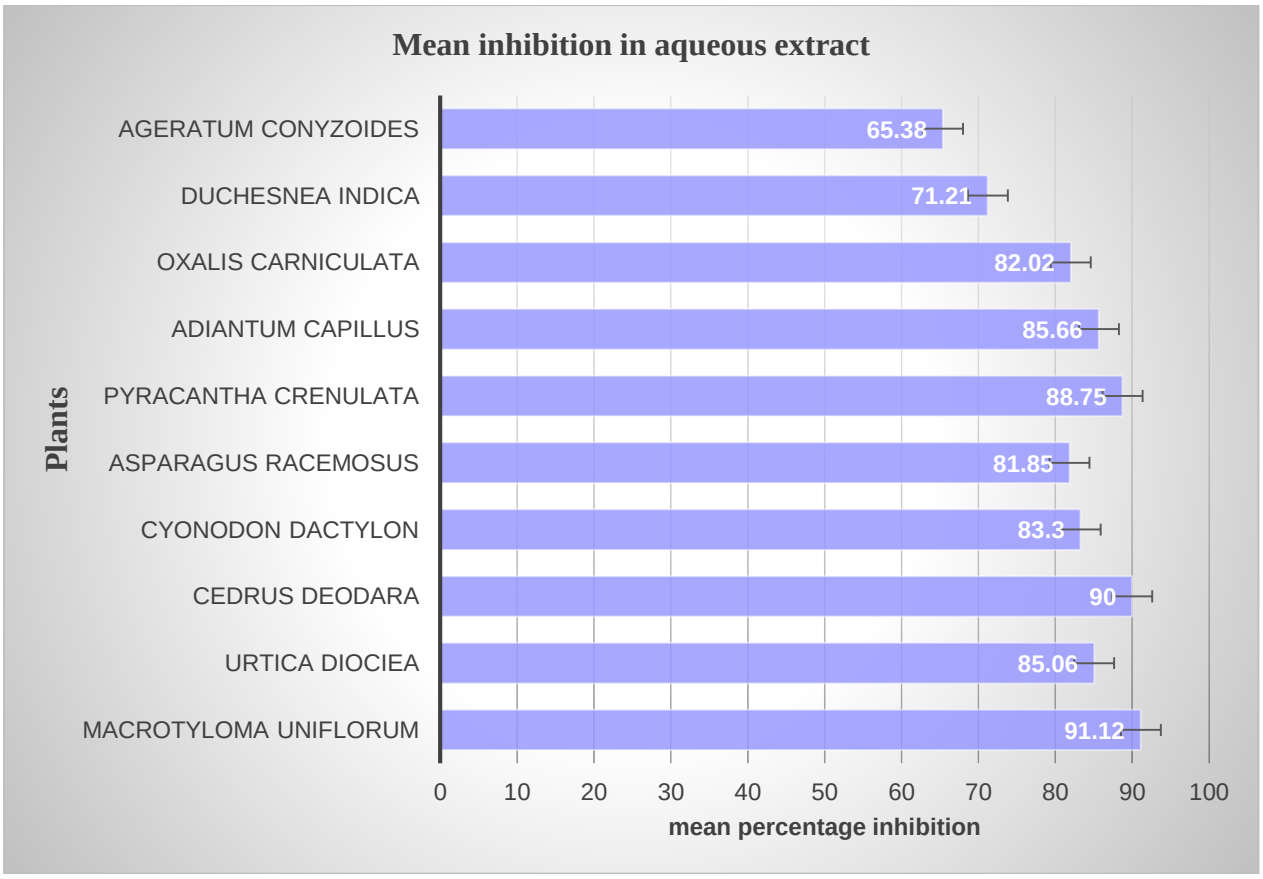

Fig: 4 **Mean anti nephrolithiasis activity in aqueous extract.**

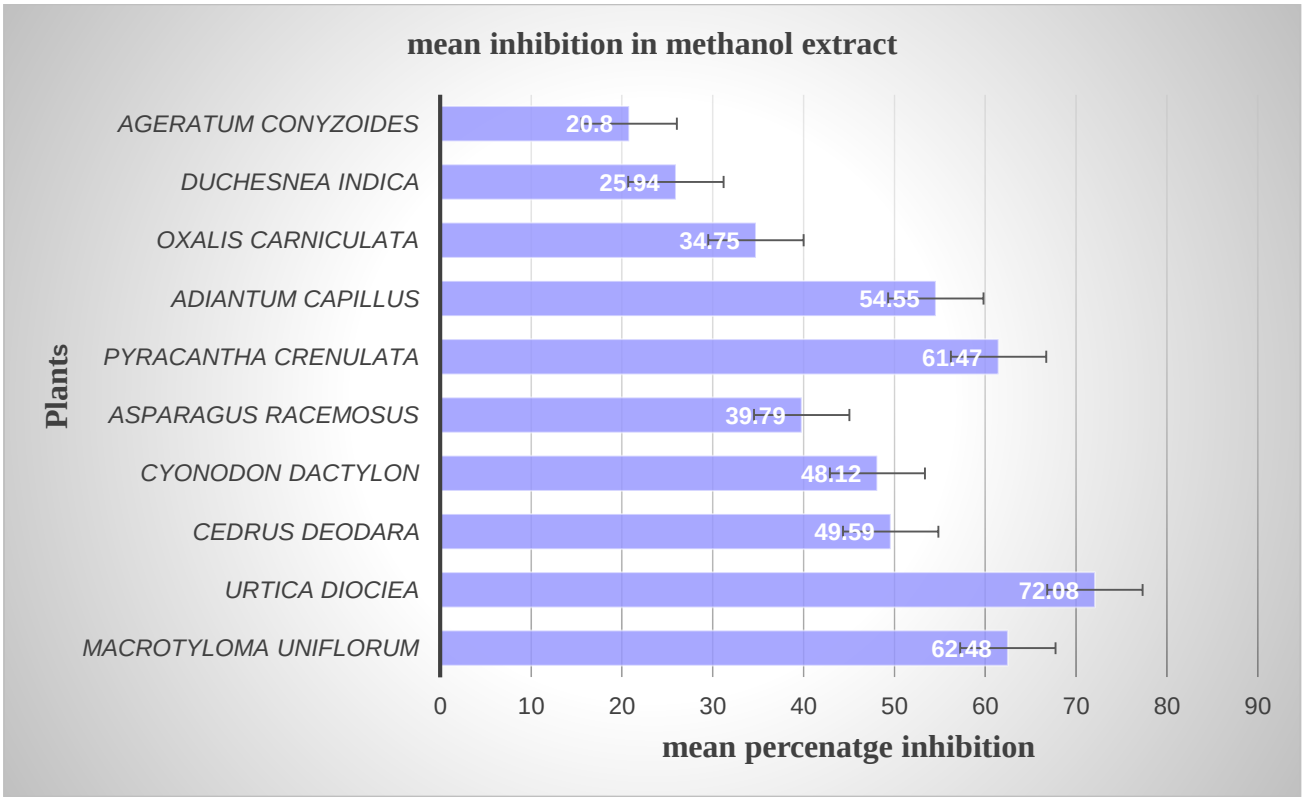

Fig: 5 **Mean anti nephrolithiasis activity in methanol extract.**

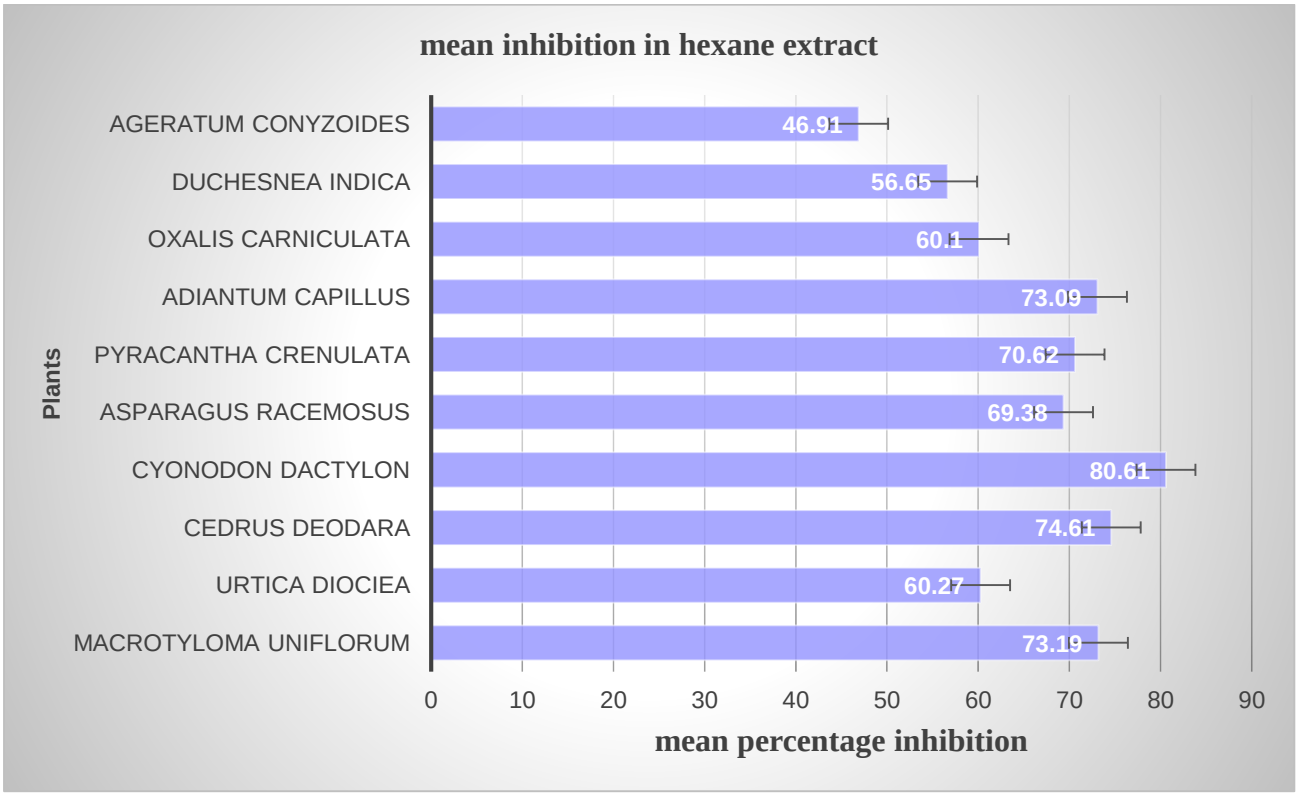

Fig: 6 Mean anti nephrolithiasis activity in hexane extract.

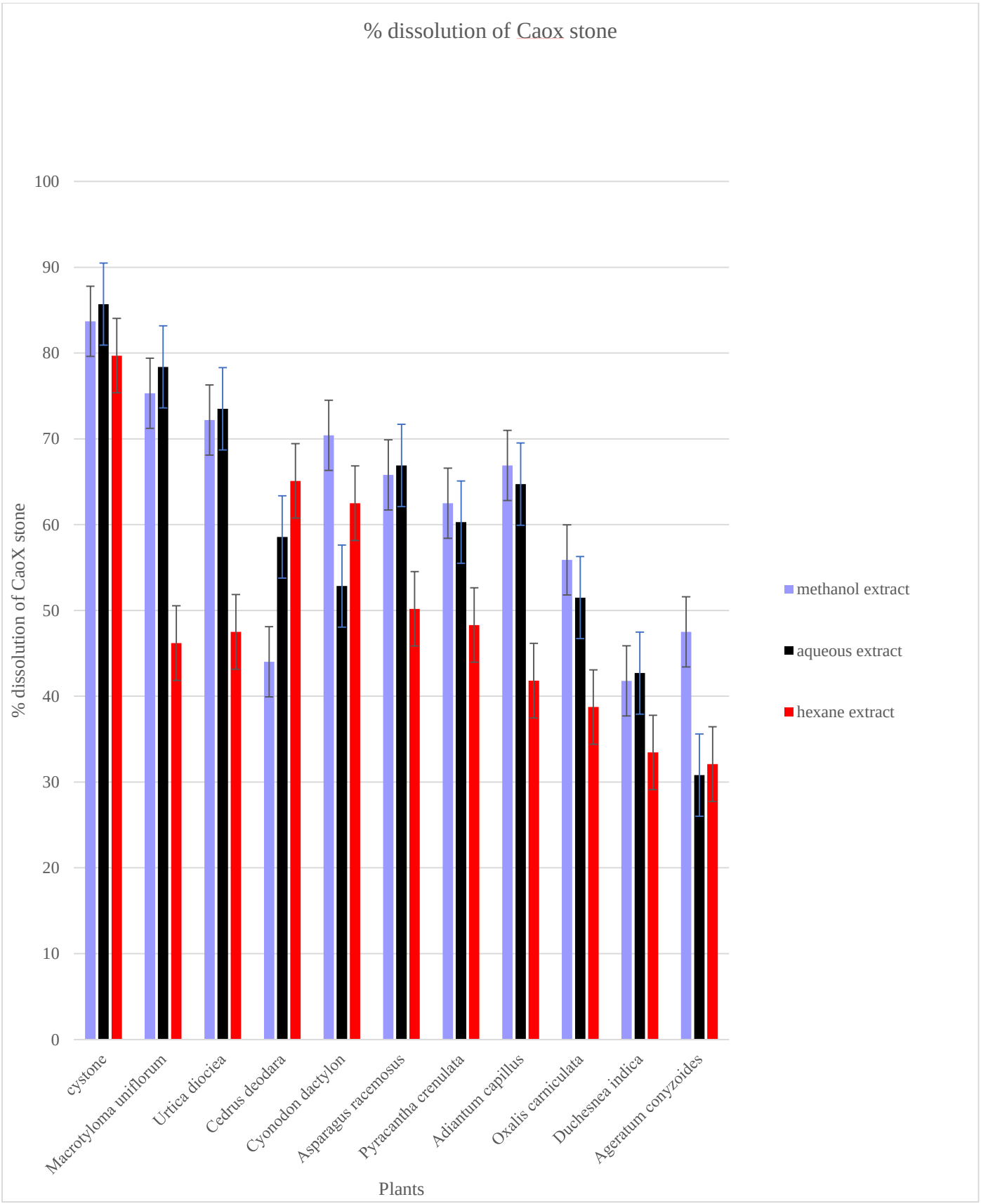

Fig 7: Percentage Dissolution of CaoX stone by different plant extract in different solvent