ejpmr, 2019,6(1), 521-526

SJIF Impact Factor 4.897

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

"INVESTIGATION THE ANTILITHIATIC POTENTIAL OF HORSE GRAM (*MACROTYLOMA UNIFLORUM*) SEEDS EXTRACTS ON EXPERIMENTAL KIDNEY STONES FROM HIGH ALTITUDE OF UTTARAKHAND (INDIA)"

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Article Received on 15/12/2018 **Article Revised on 03/01/2019** **Article Accepted on 24/01/2019**

ABSTRACT

The stones in kidney are also known as a calculus and crystal aggregation formed by dietary minerals in the urine. Lithiasis is a multifaceted progression that occurs from series of several physicochemical event including super-saturation, nucleation, growth, aggregation and retention within the kidneys. Many plant species of horse gram (*Macrotyloma uniflorum*) have been reported to posses anti-lithiatic property. In the present study aqueous, ethanolic and methanolic extracts and total seed protein of *M. uniflorum* and standard for dissolving kidney stones (collected from hospitals) and calcium oxalate crystals (*in-vitro* prepared). To confirm their anti-lithiatic potential to dissolve calcium oxalate crystals and kidney stones by an *in-vitro* model for *M. uniflorum* seeds and cystone as a standard compound collected from market. Total protein was showing significant effective in dissolving of calcium oxalate (80%) and kidney stones (65%). Aqueous fractions showed highest dissolution of stones and calcium oxalate crystals as compare to others. Aqueous fraction was more effective in dissolving calcium oxalate (85%) and kidney stones (84%). Standard-formulation Cystone was found to be more effective (87 %) when compared to others. The outcome of the study on the stone-forming minerals will provide a good indication of the risk management of stone formation. This study has given primary evidence for *Macrotyloma uniflorum* as plant which possess good anti-lithiatic property.

KEYWORD: *Macrotyloma uniflorum*, Kidney Stones, Anti-lithiatic, Calcium Oxalate, Total Protein.

INTRODUCTION

Macrotyloma uniflorum is a leguminous plant belonging to the family Fabaceae (alt. Leguminosae). It is an excellent source of iron and molybdenum and proteins (Kadam and Salunke, 1985). The seeds are ovoid and color differs from light red, brown, or black sometimes with small, scattered black spots (Blumenthal and Staples, 1993).

Macrotyloma uniflorum (Lam.) is requiring normal annual temperature ranging from 18-27°C. HG is a short day and day neutral plant maturing 120-180 days after planting. Resources deprived farmers in insignificant, drought areas of India HG late in the rainy season. HG is one of the most important pulses growing in the dry regions of the Uttarakhand. Its medicinal uses are known to Ayurveda and Uttarakhand traditional physicians for centuries. Various medicinal preparations are mainly used as a tonic, astringent, diuretic and also recommended in rheumatism, neuralgia and other several diseases. Urolithiasis is a multifaceted process that occurs from series of quite a few physicochemical event including super saturation, nucleation, growth, aggregation and retention within the kidneys. Natural foodstuffs are more useful for livings because they encourage the repair mechanism in natural way.

The present study has been undertaken to evaluate different seeds extracts (Aqueous, Ethanolic and Methanolic extracts and Total protein) of *M. uniflorum* and cystone as a standard for their possible potential to dissolve kidney stones (collected from hospitals) and calcium oxalate crystals (*in-vitro* prepared). Seeds extracts were prepared in aqueous, ethanol and methanol. Total protein was extracted by standard protocol by Sharma et al., 2018. Qualitative analysis was done for detection of various extracts and total proteins were used along with the standard cystone drug to evaluate dissolution of experimental kidney stones and human kidney stones.

MATERIAL AND METHODS

Horse gram (*M. uniflorum*) seeds were collected from local farmers of Kumaun region of Uttarakhand and stored deep freezer. The seeds were grown in field farmers. The seeds were collected and washed with

double distilled water and then taken for further analysis. Kidney stones were collected from local hospital with agreement of doctor and patient (suffer with kidney stone).

Extract preparation and Isolation of total protein

The seeds were grinded in a blender and about 20 gram of powder was extracted with double distilled water, ethanol and methanol in soxhlet extractor. All extracts were concentrated on vacuumed evaporator. All the prepared extracts were stored in reagent bottles. Total protein was extracted according to Sharma et al., 2018 with or without minor modification.

Evaluation for Anti-lithiatic Activity

The artificial urine (AU) was prepared according to the method (Burns and Finlayson, 1980; Surendra et al., 2011) that had the following recipe: NaCl: 105.5 mmol/1, Na_3PO_4: 32.3 mmol/1, $Na_3C_6H_5O_7$: 3.21 mmol/l, $MgSO_4$: 3.85 mmol/1, Na_2SO_4: 16.95 mmol/1, KCl: 63.7 mmol/1, $CaCl_2$: 4.5 mmol/1, $Na_2C_2O_4$: 0.32 mmol/1, NH_4OH: 17.9 mmol/1, and NH_4Cl: 0.0028 mmol/1. The AU was prepared fresh each day and pH adjusted to 6.0.

Preparation of experimental kidney stones (Calcium oxalate stones)

Equimolar solution of calcium chloride dihydrate was dissolved in distilled water and sodium oxalate in 10ml of 2N H_2SO_4 were allowed to react in sufficient quantity of distilled water in a beaker. The resulting precipitate was calcium oxalate (Fig. 1). The precipitate was washed with distilled water and dried at 60 ºC for 4 hours.

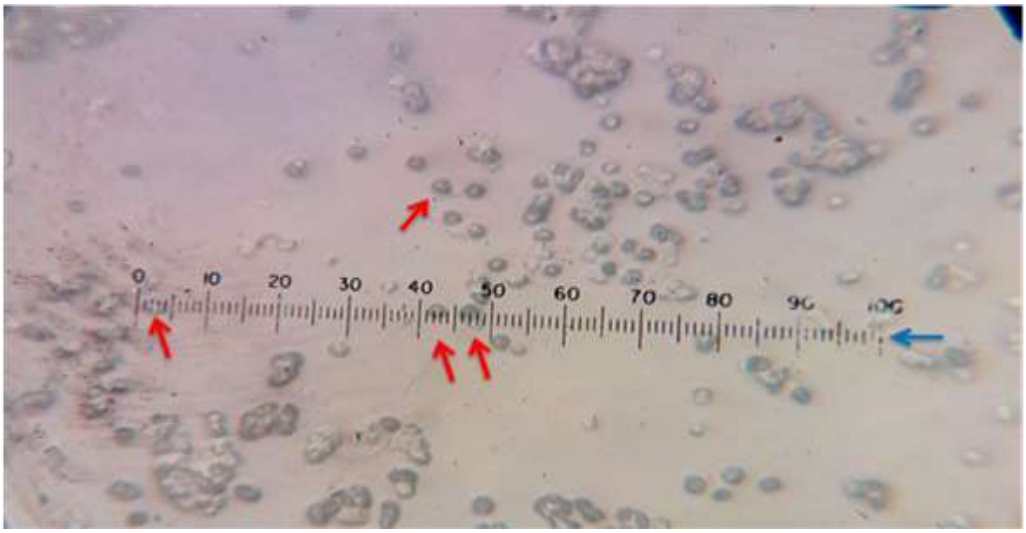

Figure 1: *In-vitro* stone preparation, size of stone is approximately 0.01mm and all are showing similar and dissimilar morphology. Stones have aggregates too. Arrow showing the scale (Blue) and crystals (Red) of calcium-oxilate (CaOx), Image resolution on 40X.

Preparation of semi-permeable membrane from farm eggs

The semi-permeable membrane of eggs is 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 (Fig. 2B). This resulted in the complete decalcification of the egg and then it was washed with distilled water (Fig. 2A). Carefully with a sharp pointer a hole was made on the top so that the contents squeeze out completely from the decalcified egg. The membrane was rinsed with distilled water and stored in refrigerator (Fig. 2C).

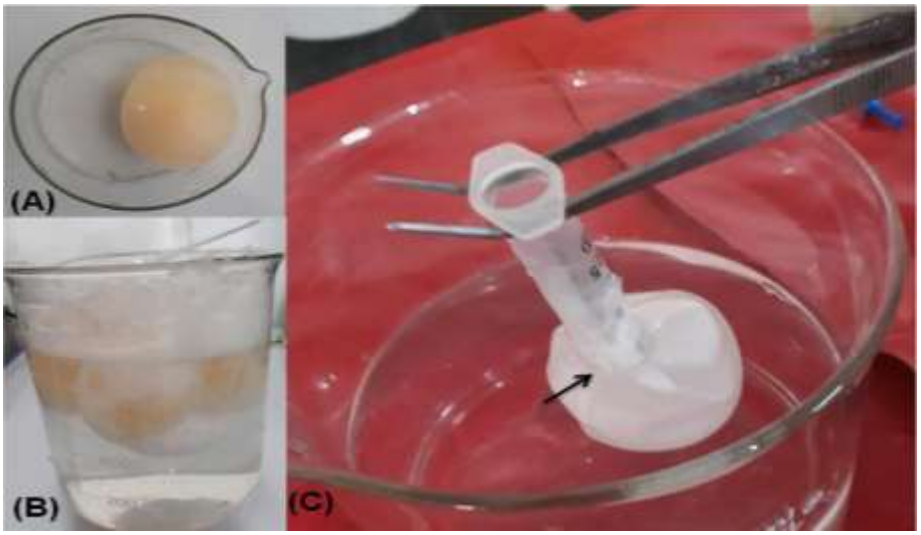

Figure 2: The separation of semi-permeable membrane of eggs and *In-vitro* Experimental model set-up to evaluate anti-urolithiatic activity of horse gram. (A): egg without outer calclous covering, (B): eggs during decalcification, (C): decalcified egg. Arrow indicates the membrane of egg.

Estimation of Calcium oxalate by titrimetry

Calcium oxalate estimation by titrimetry method was conducted according to Unnati et al., 2013 with or without minor modifications. 1mg of sample and 10mg of the extracts and standard were packed together in semi permeable membrane of eggs. The membranes were suspended in beakers containing 100ml of 0.1 M Tris buffer. 1mg of calcium oxalate served as a negative control (Fig. 2). The conical flasks of all groups were placed in a preheated incubator for about 7-8 hours. The contents of semi-permeable membrane from each group were removed into a test tube. 2 ml of 1 N sulphuric acid was added and titrated with 0.9494 N $KMnO_4$ till a light pink color end point was obtained.1ml of 0.9494 N $KMnO_4$ is equivalent to 0.1898 mg of calcium. The amount of undissolved calcium oxalate was subtracted from the total quantity used in the experiment in the beginning to determine how much quantity of calcium oxalate actually test substance(s) could dissolve.

Statistical analysis: All values were recorded as mean ± standard deviation (STDV). Microsoft Excel software was used to statistically analysis for present investigations. All values were taken in triplicate.

RESULT AND DISCUSSION

Previous analysis of horse gram indicated the presence of calcium binding proteins in seed of horse gram (Sharma et al., 2018) and on the basis of previous study the present investigation was performed *in-vitro* antilithiatic activity by comparing different extracts and total protein of *M. uniflorum* with standard (Cystone). Calcium oxalates (CaOx) were showing significant reduction in their size by applying the extracts and total protein (fig. 3). Aqueous extract, total protein and cystone gave leading in the antilithiatic drug formulation.

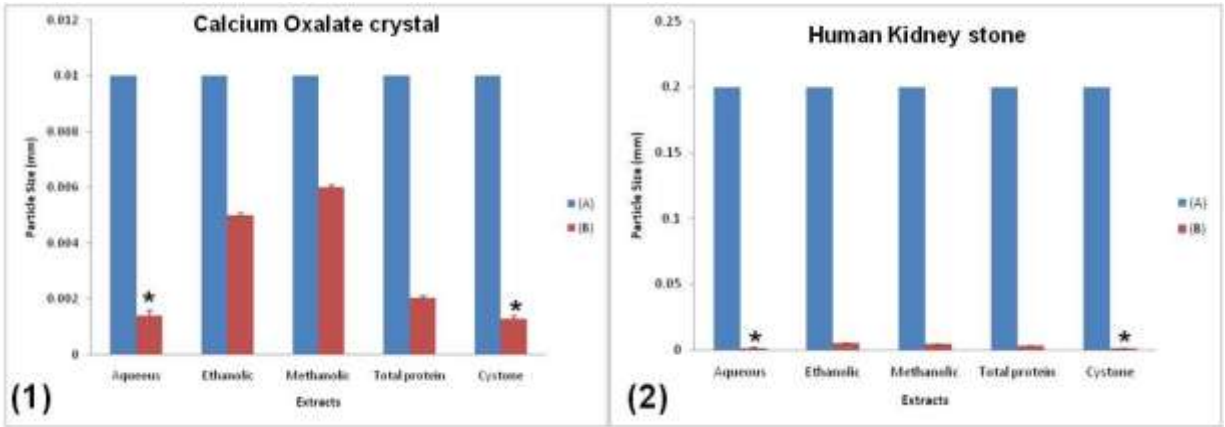

Figure 3: In vitro efficiency of horse gram extracts on Calcium oxalate crystal and human kidney stone. (1): Aqueous extract, total protein and Cystone showing significant reduction in crystal size. (2): approximately same results are come in human kidney stones. Bar (A) is before treatment and (B) is after treatment. After standardization 7hour found suitable time for size measurement of treated crystal. All values were taken in triplicate.

Microscopic Examination of *In-vitro* effect on calcium oxalate crystal of horse-gram extract

The microscopic examination clearly indicated that the significant reduction in size of CaOx crystal (Fig. 4 and Fig. 5) and human kidney stone (Fig. 6) with the treatment of horse gram extract and total protein. The present results may be milestone in the field of urolithiasis. Figure 5 showing the significant desolation of CaOx crystal when increase the total protein amount. Aqueous extract of horse gram also showing the drastic desolation of Human kidney stones (Figure 6).

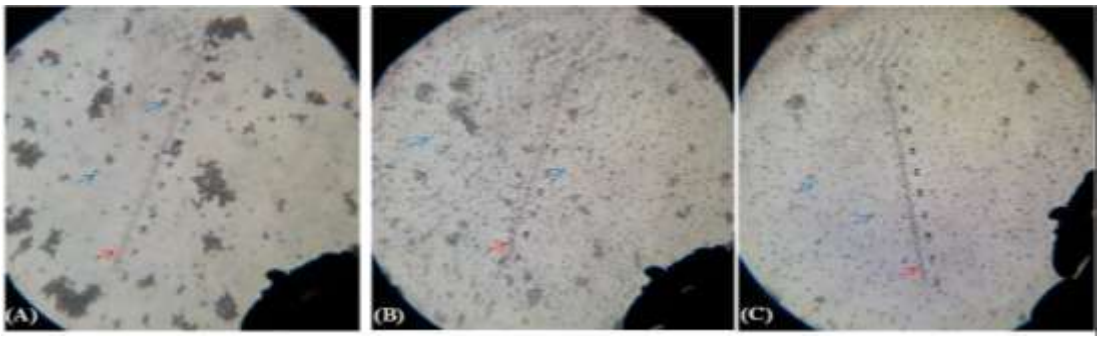

Figure 4: *In-vitro* effect of horse-gram extract on calcium oxalate crystal stones having aggregates. (A): control; (B): having 1mg/ml extract treatment on CaOx crystals; (C): having 2mg/ml extract treatment on CaOx crystals. Arrow showing the scale (Blue) and crystals (Red) of calcium-oxilate (CaOx), Image resolution on 40X.

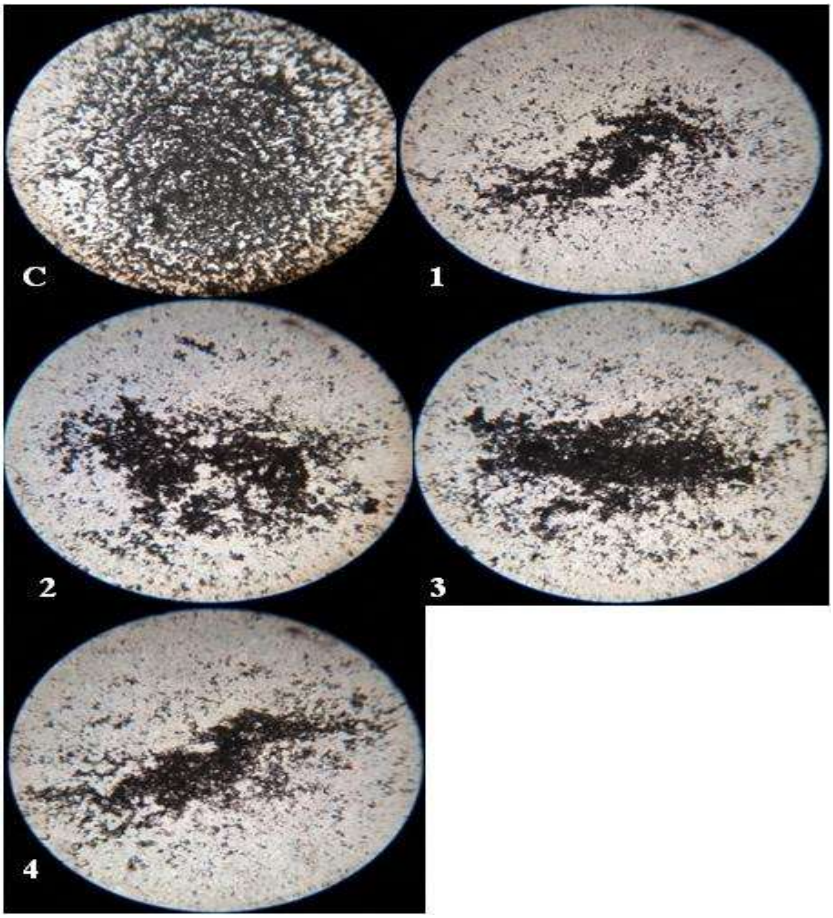

Figure 5: Microscopic examination to *In-vitro* effect of horse-gram's total protein on calcium oxalate crystal stones having aggregates. (C): control (without any treatment); (1): having 1µg/ml; (2): 2µg/ml; (3): 3µg/ml; (4): 4µg/ml total protein treatment on CaOx crystals. Image resolution on 20X.

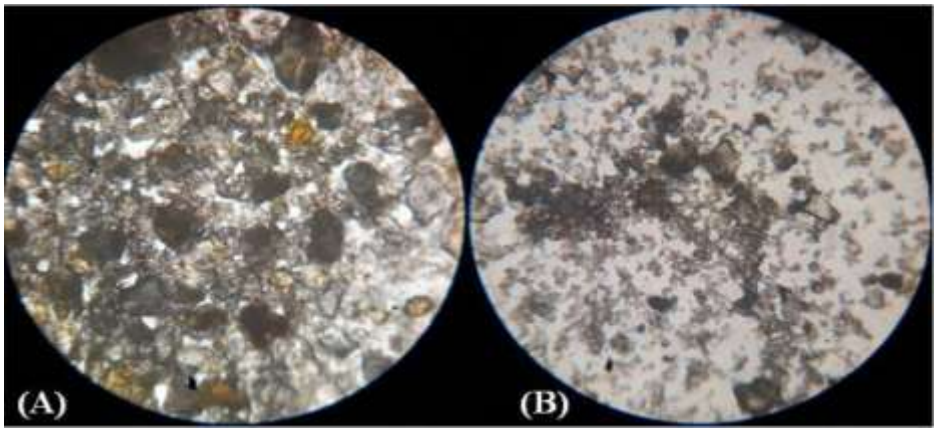

Figure 6: *In-vitro* effect of horse-gram aqueous extract on Human kidney stone (Crushed) having aggregates. (A): control (none treated); (B): having 10mg/ml of aqueous extract on crystals. Image resolutions on 40X.

Present study evaluates that anti-lithiatic activity of different extracts of *M. uniflorum* seeds and total protein. *In vitro* crystallization systems were widely used for many experimental purposes in antilithiasis research (Khan, 1997). The examination of the urinary chemistry with respect to the stone-forming minerals will provide a good sign of the danger of stone configuration. The investigation of the chemistry of urine with respect to the formation of mineral stone will provide a good sign of the risk of stone formation (Unnati et al., 2013). All urinary stones are in partially composed of organic matrix; however, there has been much debate as to the importance of this material in urinary stone formation. It has been suggested that matrix has an active role in stone formation and may act as a binding or cementing material that is necessary for crystal aggregation and growth (Martin et al., 1981). In a study by Chaitanya et al., 2010 the alcoholic extract of *M.uniflorum* shows better anti urolithiatic activity than aqueous extract but the present study showing aqueous extract better than others.

CONCLUSION

The outcome of the present investigation will be helpful for formulation of antilithiatic drug by horse gram. Urolithiasis has been performed for *M. uniflorum* seeds extracts, total protein and cystone (Himalayan Health Care) and performed work by using *In-vitro* Antilithiatic model for calculating present reduction in size of CaOx stone and human kidney stone. Aqueous extract and total protein were gave higher present reduction in size of both types of crystal. Present investigation has given evidence for *Macrotyloma uniflorum* as plant which possess good anti-lithiatic potency.

ACKNOWLEDGEMENT

The authors wish to acknowledge the Science Engineering and Research Board, Department of Science and Technology, New Delhi, Govt. of India. The authors are also thankful to farmers of Uttarakhand for providing the seed samples of the cultivar analyzed in present study.

Funding statement

National post doctoral fellow in Science Engineering and Research Board, Department of Science and Technology, New Delhi, Govt. of India. Vide letter no. PDF/2016/001883.

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