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EVALUATION OF POLYHERBAL EXTRACTS FOR *IN-VITRO* ANTIUROLITHIATIC ACTIVITY USING BIOENHANCER

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ABSTRACT

Leaves of *Kalanchoe pinnata*, core stem of *Musa paradisiaca* and corn silk *of Zea mays* are evaluated and reported by the researchers for their antiurolithiatic activity but the activity of combination of these drugs in presence of bioenhancer like *Pepper nigrum* was not explored yet. The current study was design to evaluate the effect of combination of the plants with known antiurolithiatic activity with bioenhancer (piperine from pepper) by *in-vitro* model. Pepper enhances the bioavailability of structurally and therapeutically different drugs, either by increasing the absorption or by delaying the metabolism of the drug or by a combination of both processes. The objective of the proposed study was to assure the antiurolithic efficacy of indigenous herbs using *in-vitro* assays, to study alterations in activity or efficacy produced by application of bioenhancer and to examine the herbal combination for possible synergy and simultaneous determination of optimum dose or concentration of the drug as well as the bioenhancer for maximum potency. The combination was studied in presence and absence of bioenhancer & standard (Cystone) to check if any improvement in efficacy was observed which can reduce the dose of the polyherbal combination.

KEYWORDS: Bioenhancer, Antiurolithic, Urolithiasis.

INTRODUCTION

Nephrolithiasis and urolithiasis are stones within the renal collecting system (nephrolithiasis) or elsewhere in the collecting system of the urinary tract (urolithiasis). Calculi often form and accumulate in the renal pelvis and calyces. Stones vary in composition, depending on individual factors, geography, metabolic alterations and the presence of infection. For

unknown reasons, renal stones are more common in men than in women. They vary in size from gravel (< 1 mm in diameter) to large stones that dilate the entire renal pelvis. While they may be well tolerated, in some cases, they lead to severe hydronephrosis and pyelonephritis. They can also erode the mucosa and cause hematuria. Passage of a stone into the ureter causes excruciating flank pain, renal colic. Until recently, most kidney stones required surgical removal, but ultrasonic disintegration (lithotripsy) and endoscopic removal are now effective. (Rubin's pathology Clinicophathologic Foundation of Medicine 6th Edition, Lippincott Williams & Wilkins. pp. 800, 801)

Most (75%) renal stones are calcium complexed with oxalate or phosphate, or a mixture of these anions. Calcium oxalate is more common, these stones are hard and occasionally dark. Calcium phosphate stones tend to be softer and paler. It is estimated that 12% of world population experiences renal stone disease with a recurrence rate of 70-80% in males and 47-60% in females.[1,2] Urinary calculi are the third most common affliction of the urinary tract which are exceeded by the urinary tract infections and prostate diseases.

Currently a number of allopathic drugs are used for treatment of urolithiasis. A combination of physical methods such as ultrasound and laser in combination with allopathic medicine are widely used. In the present scenario, the demand for herbal products is growing exponentially throughout the world and major pharmaceutical companies are currently conducting extensive research on plant materials for their potential medicinal value.[4] A number of plants are known for their utility in folklore medicine as Urolithics. The examples of these include *Kalanchoe pinnata*, *Musa paradisiaca* and *Zea mays*. The objective of current work is to study three indigenous drugs viz. *Kalanchoe pinnata*, *Musa paradisiaca* and *Zea mays* in combination using a bioenhancer like *Pepper nigrum*, for their potential as antiurolithiatic agents.[5,6,7,8]

Till date a number of reports on use of herbal medicines as antiurolithiatic agent have greatly increased. Newer methods and chemical entities along with herbal medicines are being screened for their efficacy. With the help of indigenous knowledge and modern technology we can generate newer agents and validate the folklore medicine. This in turn will help in generation of Intellectual property and broaden the spectrum of medicines currently available for therapy.[12,13]

Exploitation of Indian flora for development of alternative therapy will be beneficial in making the treatment available to the people who cannot afford surgery or expensive allopathic treatments. Number of polyherbal formulations containing indigenous herbs has already been patented and some are in the process for therapy as antiurolithics. Development of a polyherbal combination with proven efficacy shall further nurture the process of development of alternative medicine for urolithiasis.[14]

EXPERIMENTAL WORK

Experimental design

Group I: Negative Control (1mg Calcium oxalate / Calcium phosphate)

Group II: 1 mg Calcium oxalate / Calcium phosphate + 10 mg standard (Cystone)

Group III: 1 mg Calcium oxalate / Calcium phosphate + 10 mg extract of each *M. paradisiaca, B. pinntum, Zea mays* and *P. nigrum* + standard (Cystone)

Group IV: 1 mg Calcium oxalate / Calcium phosphate + 10 mg extract of each *M. paradisiaca, B. pinntum, Zea mays* and *P. nigrum*.

Group V: 1 mg Calcium oxalate / Calcium phosphate + 10 mg extract of each *M. paradisiacal, B.pinntum and Zea mays.*

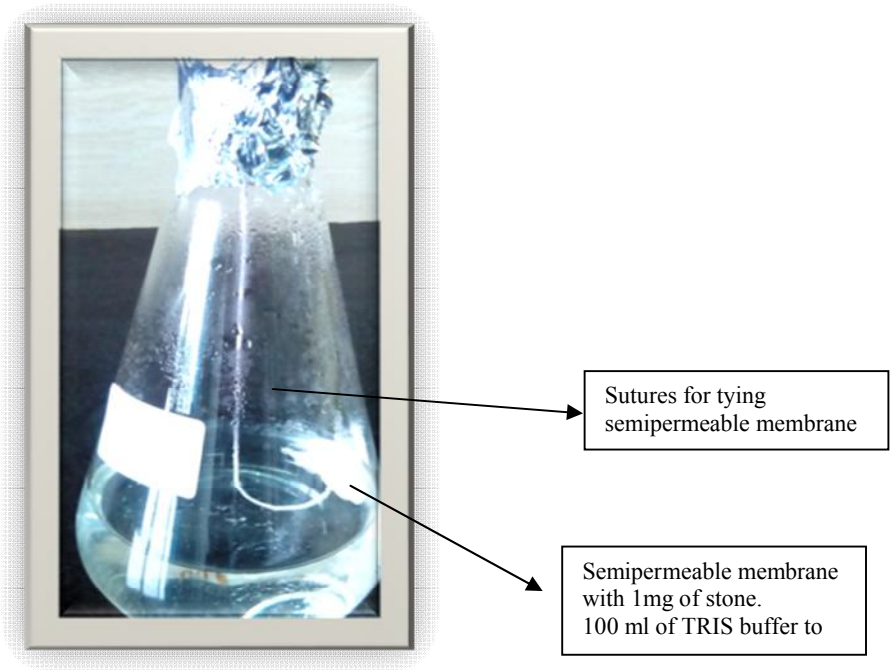

Fig 1: *In-vitro* experimental model set-up to evaluate antiurolithiatic activity.

Procurement and Authentication of Plant Material

The drug was procured from medicinal plant garden of the Institute. Black pepper and corn silkwas purchased from local market. Plants procured were authenticated by Dr. Vinayak Naik, Senior Botanist, Piramal Health Care. The authenticated plant material was then be used for further studies.

Extraction by continuous hot percolation

Dried plant material was coarsely powdered and subjected to continuous hot extraction by using hydroalcohol as solvent in Soxhlet Extractor.

***In –vitro* evaluation of antiurolithiatic activity**

Preparation of the Semi-permeable membrane from farm eggs

The outer calcified shell was removed chemically by placing the eggs in 2M HCl overnight, which cause complete decalcification. Further, outer surface of decalcified eggs was washed with distilled water. Then with a sharp pointer; a hole was made carefully on the top. The contents were squeezed out completely from the decalcified egg. Then the membrane was washed thoroughly with distilled water and placed in ammonia solution, in a moistened condition. It was then rinsed with distilled water and stored in refrigerator at a pH of 7- 7.4.

Estimation of calcium oxalate by Titrimetry

Preparation of Calcium oxalate by homogenous precipitation

10 ml each, equimolar solution of Calcium Chloride Dihydrate (A.R) in distilled water and Sodium Oxalate (A.R) in 2N H_2SO_4 was allowed to react in a beaker. The resulting precipitate is Calcium Oxalate which was then freed from traces of sulfuric acid by treatment with ammonia solution. Finally it was washed with distilled water and dried at a temperature 60 °C for 4 hours.

Method

The studies were carried out in five groups as per experimental design. One group served as negative control (containing only 1 mg of calcium oxalate). Exactly 1 mg of the calcium oxalate, 10 mg hydroalcoholic extract of each drug and 10 mg standard Cystone were weighed and packed in semi permeable membrane by suturing. They were suspended in a conical flask containing 100 ml 0.1 M TRIS buffer. The conical flasks of all groups were placed in an incubator, pre heated to 37^0 C for 2 hours, for about 7-8 hours. The contents of semi permeable membrane from each group were taken into test tubes. 2 ml of 1 N sulfuric

acid was added to each test tube and titrated with 0.9494 N $KMnO_4$ till a light pink color end point obtained. 1ml of 0.9494 N $KMnO_4$ is equivalent to 0.1898 mg of Calcium. Percentage dissolution of calcium oxalate in various groups was calculated.

Table 1: Percent of Calcium oxalate dissolution by various groups under study.

Groups as per Experimental Design	Vol. of Standard $KMnO_4$	Wt. of Calcium Estimated(mg)	Wt. of Calcium Reduced (mg)	% Dissolution
I	4.4	0.8448	0.1552	15.52
II	2.5	0.48	0.3648	36.48
III	2.3	0.4416	0.4032	40.32
IV	2.9	0.5568	0.288	28.80
V	3.1	0.5952	0.2496	24.96

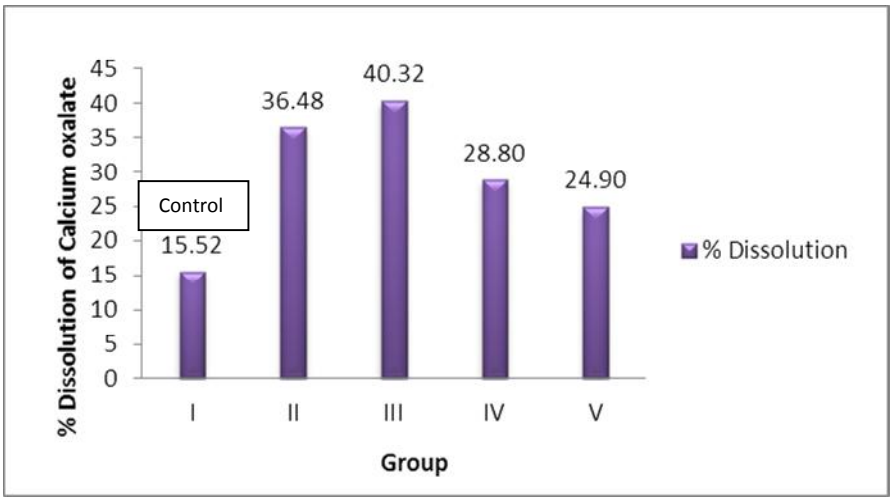

Figure 2: Percentage dissolution of Calcium oxalate by various groups under study.

Estimation of Calcium phosphate by Colorimetry

Preparation of Calcium phosphate by homogenous precipitation

10 ml each, equimolar solution of Calcium Chloride Dihydrate (A.R) in distilled water and Disodium Hydrogen Phosphate (A.R) in 2N H_2SO_4 was allowed to react in a beaker. The resulting precipitate of Calcium Phosphate was freed from traces of sulfuric acid by treating with ammonia solution. Finally it was washed with distilled water and dried at a temperature 60^0 C for 4 hours.

Preparation of Molybdate-sulphuric acid reagent

It was prepared by mixing 2 parts of 5% w/w solution of Sodium molybdate (A.R), 1 part of 10 N sulfuric acid and 1 part of distilled water.

Preparation of reducing solution

1 g of *p*-Phenylenediamine was dissolved in 100 ml of 3 % w/w solution of Sodium Bisulfite to get the required solution.

Method

The studies were carried out in five groups as per experimental design. One group served as negative control (containing only 1 mg of calcium phosphate). Exactly 1 mg of the calcium phosphate, 10 mg hydroalcoholic extract of each drug and 10 mg standard Cystone were weighed and packed in semi permeable membrane by suturing. This was allowed to suspend in a conical flask containing 100 ml 0.1 M TRIS buffer. The conical flasks of all groups were placed in incubator, pre heated to 37^0 C for 2 hours, for about 7-8 hours. The contents of semi permeable membrane from each group were taken in a test tube. 2 ml of 1 N sulfuric acid, 2.5 ml of Molybdic-sulphuric acid reagent, and 1 ml of reducing solution was added. Volume was made to 10 ml using distilled water. Standard dilutions of calcium phosphate were prepared, (200, 400, 600, 800 and 1000 µg/ml) containing 2.5 ml of Molybdic-sulphuric acid reagent, 1 ml of reducing solution. Volume was made up to 10 ml using distilled water. The optical densities of standard dilutions and for the groups under study were measured by colorimeter at 600-750 nm. The undissolved calcium phosphate was determined from the standard calibration curve by extrapolation. The results of the various groups were interpreted as percentage dissolution.

Table 2: Dissolution of Calcium phosphate by various groups under study.

Standard	Molybdic H_2SO_4 reagent	Reducing Solution	Distilled Water (q.s.)	Optical Density
200 µg/ml	2.5 ml in each	1 ml in each	Up to 10 ml in each	0.04
400 µg/ml				0.11
600 µg/ml				0.16
800 µg/ml				0.23
1000 µg/ml				0.27
Groups as per Experimental Design	**Molybdic H_2SO_4 reagent**	**Reducing Solution**	**Distilled Water (q.s.)**	**Optical Density**
I	2.5 ml in each	1 ml in each	Up to 10 ml in each	0.21
II				0.09
III				0.07
IV				0.13
V				0.15

Table 3: Dissolution of Calcium phosphate by various groups under study.

Groups as per Experimental Design	Optical Density	Wt. of Calcium Reduced (mg)	% Dissolution
I	0.21	0.23	23
II	0.09	0.67	67
III	0.07	0.74	74
IV	0.13	0.52	52
V	0.15	0.45	45

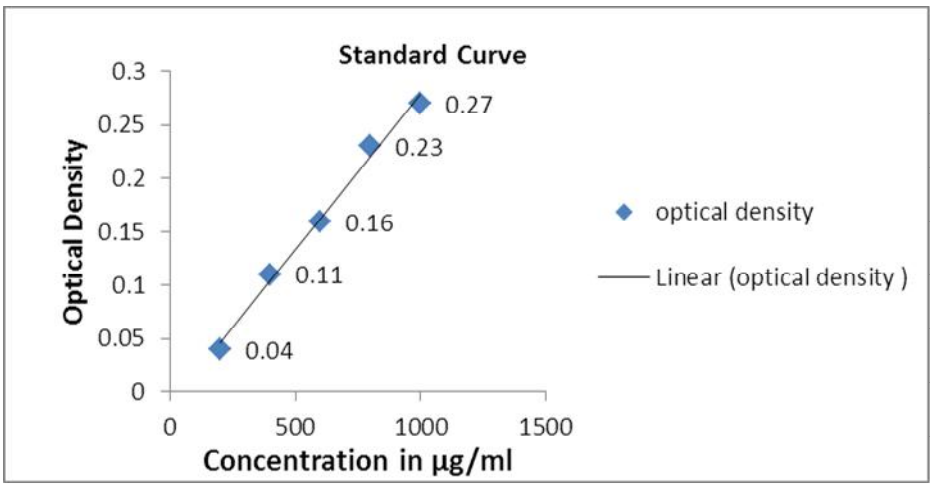

Figure 3: Standard calibration curve of Calcium phosphate by colorimetry.

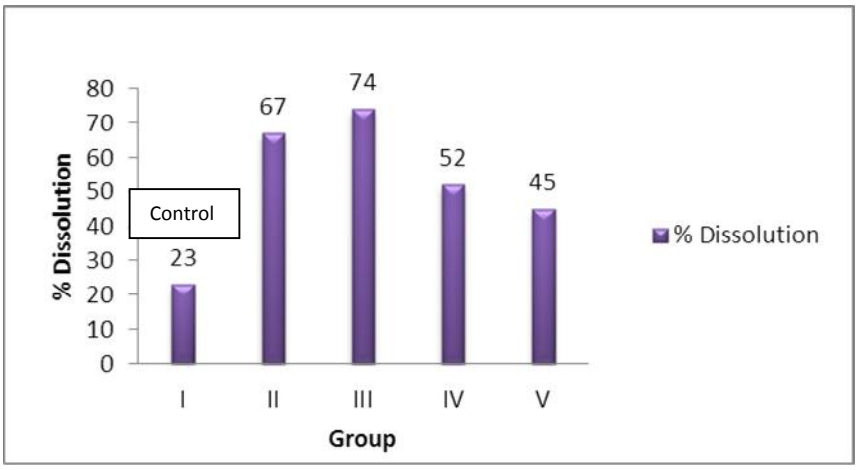

Figure 4: Percentage dissolution of Calcium phosphate by various groups under study.

RESULTS AND DISCUSSION

Development of a polyherbal combination with proven efficacy will further nurture the process of development of alternative medicine for urolithiasis.

Leaves of *Kalanchoepinnata*, core stem of *Musa paradisiaca*and corn silk *of Zea mays* are individually evaluated and reported by the researchers for their antiurolithiatic activity but the

activity in combination of these drugs in presence of bioenhancer like *Piper nigrum* has not been explored yet. The current studies were designed to evaluate the effect of combination of the plant extracts with known antiurolithiatic activity with bioenhancer (piperine from Black Pepper) by *in-vitro* model.

Group III showed the maximum dissolution of calcium oxalate (40.32%) and calcium phosphate (74.0%) compared to control (15.52 % & 23.0% calcium oxalate & calcium phosphate respectively). This group contained extracts under studies with standard (Cystone) and bioenhancer (*P. nigrum*). There was significant increase in the dissolution (24.8% & 51.0% calcium oxalate & calcium phosphate respectively) of both the stones in presence of natural bioenhancer as compare to Group II (36.48% & 67% calcium oxalate & calcium phosphate respectively) containing only standard (Cystone) and Group V (24.90% & 45% calcium oxalate & calcium phosphate respectively) containing only extracts under study.

Compare to group V, Group IV has showed increased dissolutionof calcium phosphate (7%) and calcium oxalate (3.9%) this may be due to presence of bioenhancer in Group IV.Group V has showed least activity as compare to other group against both the stone.All the Groups under study have shown antiurolithic activity and also indicate that the polyherbal extract if given with bioenhancer showed enhanced effect on dissolution of both types of stones.

CONCLUSION

A newer combination utilizing potential antiurolithic agent in presence of a naturally occurring bioenhancer was successfully evaluated for its efficacy. The combination was studied in presence and absence of bioenhancer as well as in presence & absence of standard (Cystone) to check if any improvementin efficacy was observed which can reduce the dose of the polyherbal combination. From the results it is observed that there is increase in efficacy of various extracts in presence of bioenhancerwhich can further be extrapolated to *in vivo* models. Development of polyherbal formulation in combination with natural bioenhancer will aidin reducing the dose. These findings can be applied to modern system of medicine by incorporating it into a dosage form which will make it more acceptable for patients.

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