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Antiuro lithiatic Activity of *Casaurina equisetifolia* Leaf Mediated Nanoparticles

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Abstract: Urolithiasis is a complex process that occurs from series of several physiochemical event including super-saturation, nucleation, growth, aggregation and retention with the kidneys. Data from *in vitro*, *in vivo* and clinical trials reveals that phytotherapeutic agents could be useful as either alternative or an adjunct therapy in the management of urolithiasis. *Casaurina equisetifolia* have been reported to possess antiuro lithiatic property by various folk lore practitioners. The *in vitro* antiuro lithiatic study of the whole plant of *C. equisetifolia* through titrimetric and turbidity method was performed to check their potential in dissolving calcium oxalate crystals using Neeri as a standard compound. The aqueous extract and alcoholic extract of Gamma treated samples showed relatively higher dissolution of 70% of stones. Alcoholic extract of NPs and Gamma treated samples showed more dissolution than aqueous extract.

Keywords: Urolithiasis, *In vitro*, Gamma radiation, Titrimetric, Turbidity, Ultrasonication, Cobalt-60

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Introduction

Urolithiasis is the condition where urinary calculi are formed or located anywhere in the urinary system or the process of formation of stone in kidney, bladder or ureter. Calculi, is an aggregation of solute materials from urine such as calcium, oxalate, phosphate and uric acid which form stone. It is a serious, debilitating problem in

all societies throughout the world, affecting approximately 12% of the population and men are three times more prone than women. It is more prevalent between the ages of 20 and 40 in both sexes. Etiology is multifactorial and is strongly related to dietary lifestyle habits or practices. Increased rates of hypertension and obesity, also

contribute to an increase in stone formation. Surgical intervention and pain management are the main treatment procedures followed in this disease. The major part of the population is trying to find alternatives to modern medicines because of their side effects. Ayurveda, an indigenous Indian system of medicine, offers vast scope for the successful treatment of urinary tract problems including urolithiasis. Traditional system of medicine uses many herbs in different dosage forms with success stories without any side effects. But exact mode of action, evident facts are yet to be derived, to popularize such cost effective safe herbal drug practices. *Casuarina equisetifolia* is commonly called as whistling pine. The whole plant is used in the form of decoction to dissolve stones with different adjuvants. In the present study an effort has been made to evaluate anti-urolithiatic activity of *Casuarina equisetifolia* by titrimetric method.

Materials and Methods

Preparation of leaf extract:

The mature, undamaged, and disease-free leaves of *Casuarina equisetifolia* were collected from campus of Dr. Babasaheb Ambedkar Marathwada University, Aurangabad, Maharashtra, India. The plant leaves were washed thoroughly with double distilled water and dried under shade. The dried leaves were segregated, pulverized by a mechanical grinder, and stored in a plastic box at room temperature. The crude extract of the plant material was prepared by mixing 1g of plant powder with 100 ml of double distilled water. Then it was ultrasonicated at three different frequencies (3 Hz frequency for 15 min, 5 Hz frequency for 10 min and 10 Hz frequency for 5 min, with the Multi Frequency Ultrasonic Inferometer) (Table 1). The crude extract was then filtered using Whatman filter paper number 1 to get a clear solution which was used as the stock solution (Sasidharan *et al.*, 2018).

Synthesis of silver nanoparticle:

Under room temperature, 10 ml of leaf extract stock solution was added to 90 ml of 1 mM silver

nitrate solution. After mixing thoroughly, the solution was exposed to the sunlight for 5 min. The solution became yellow to brown. The colored solution was centrifuged at 10,000 rpm for 15 minutes to obtain pellets. To produce nanoparticles free of any biological material present in synthesized silver nanoparticles, the pellet was washed with ethanol and dispersed in sterile distilled water (Kumar *et al.*, 2007)

Uv visible spectroscopy:

The size of the silver nanoparticles is controlled using reducing agent sodium borohydride and characterized using UV-visible spectroscopy technique. This method under the frame work of Mie theory is used to determine particle size and size distribution. The UV-vis absorbance is used to characterize the kinetics of formation and final colloid stability (Fig. 1). Using chemical reduction technique silver nanoparticles have been synthesized having size < 5 nm with controllable (Desai *et al.*, 2012).

Ultrasonication:

A simple, inexpensive ultra-sonication method was used to synthesize quasi spherical silver nanoparticles (AgNPs) with an aqueous extract from *Casuarina equisetifolia*. This method has the advantages of being completely eco-friendly and allows increased reaction rates, uniform dispersal of the nanoparticles in liquids, and effective breaking of aggregates. Biomolecules present in plant extracts are often used to reduce metal ions to nanoparticles in a single-step green synthesis route. Different probes are used for samples i.e. 3, 5, 10 htz . Sample for 3 htz treated for 15 min, sample for 5 htz treated for 10 min, sample for 10 htz treated for 5 min (Fig. 2) (Sun and Xia, 2002).

Gamma Irradiation Treatment (Co60):

Decalcified eggs were irradiated with gamma rays (source 60Co) for 10, 15 and 20 min of incubation (Table 2). In the same experiment, there were included the same number of decalcified eggs unexposed to gamma-radiation and served as controls. Non-irradiated egg shells were retained

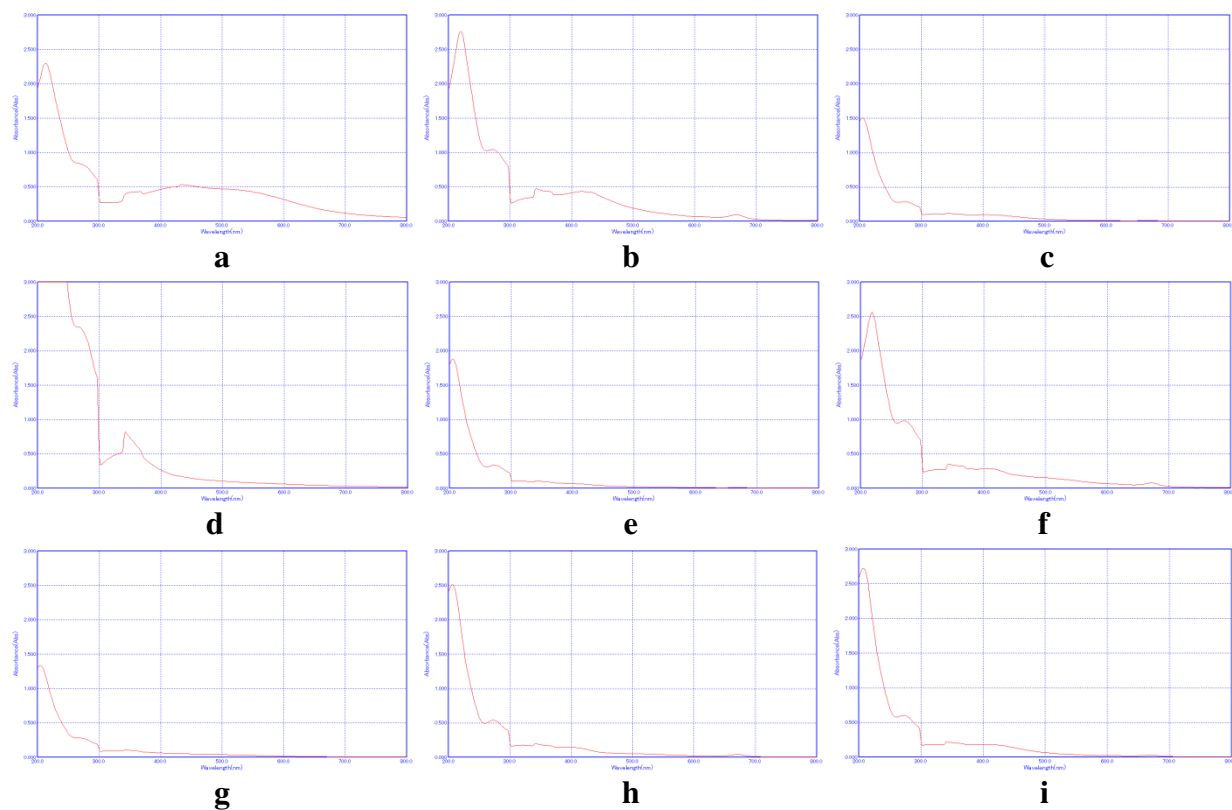


Fig. 1: Ultraviolet-visible spectroscopy analysis. (a) 3 htz distilled water AgNPs; (b) 3 htz ethanol AgNPs; (c) 3 htz methanol AgNPs; (d) 5 htz distilled water AgNPs; (e) 5 htz ethanol AgNPs; (f) 5 htz methanol AgNPs; (g) 10 htz distilled water AgNPs; (h) 10 htz ethanol AgNPs; (i) 10 htz methanol AgNPs.

Table 1: Results of dissolution studies of calcium oxalate by NPs plant extract *Casuarina equisetifolia*

Treated with Ultrasonic Inferometer	KMnO ₄ wt/v	Calcium estimated by weight	calcium reduced by weight	Per cent dissolution of calcium
Control	0.12	0.0227	0	
Neeri	0.02	0.019	0.0037	83.7
3 htz d/w extract	0.03	0.017	0.0057	74.89
3 htz met. extract	0.02	0.019	0.0037	83.7
3 eth. extract	0.02	0.019	0.0037	83.7
5 probe d/w extract	0.04	0.015	0.0212	66.96
5 probe met. extract	0.02	0.019	0.0037	83.7
5 probe eth. extract	0.02	0.019	0.0037	83.7
10 probe d/w extract	0.04	0.015	0.0212	66.96
10 probe met. extract	0.02	0.019	0.0037	83.7
10 probe eth. extract	0.03	0.017	0.0057	74.89

d/w = distilled water; met. = methanolic; eth. = ethanolic

Table 2: Results of dissolution studies of calcium oxalate by Gamma radiation treated plant extract *Casuarina equisetifolia*

Gamma Irradiated sample	KMnO ₄ wt./vol	Weight of Calcium estimated	Weight of calcium reduced	Per cent of dissolution rate
Control	0.12	0.0227	0	-
Neeri	0.02	0.019	0.0037	83.7
C. equ. 20 min d/w extract	0.03	0.017	0.0057	74.89
C. equ. 20 min Met. extract	0.04	0.015	0.0212	69.69
C. equ. 20 min Eth. extract	0.04	0.015	0.0212	70.11
C. equ. 15 min d/w extract	0.04	0.015	0.0212	66.96
C. equ. 15 min Met. extract	0.03	0.017	0.0057	74.89
C. equ. 15 min Eth. extract	0.02	0.019	0.0037	83.7
C. equ. 10 min d/w extract	0.03	0.017	0.0057	74.89
C. equ. 10 min Met. extract	0.02	0.019	0.0037	83.7
C. equ. 10 min Eth. extract	0.03	0.017	0.0057	74.89

d/w= distilled water; Eth. = Ethanolic; C. equ. = *Casuarina equisetifolia*; Met. = Methanolic

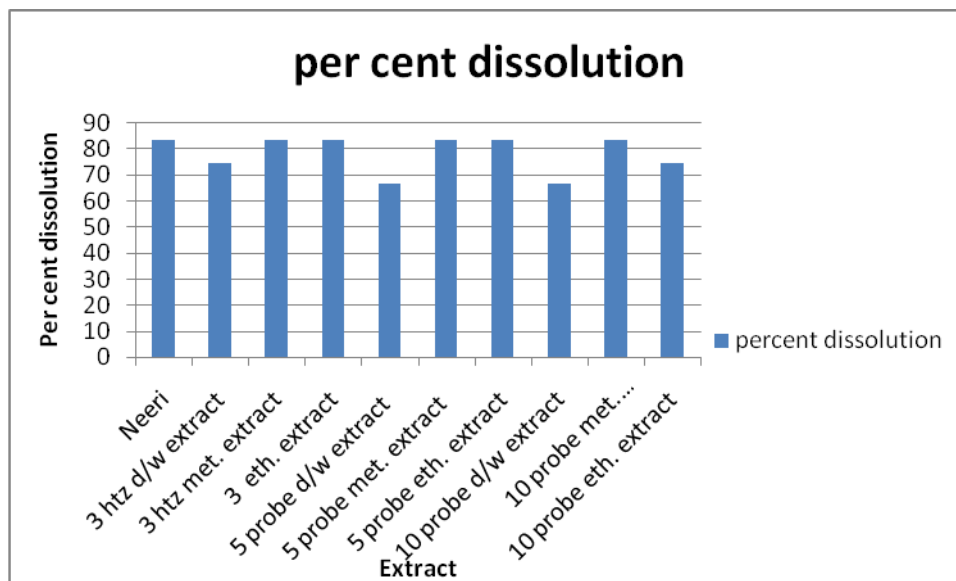


Fig. 2: Percentage dissolution of calcium oxalate by ultra sonicated *Casuarina equisetifolia* extract groups.

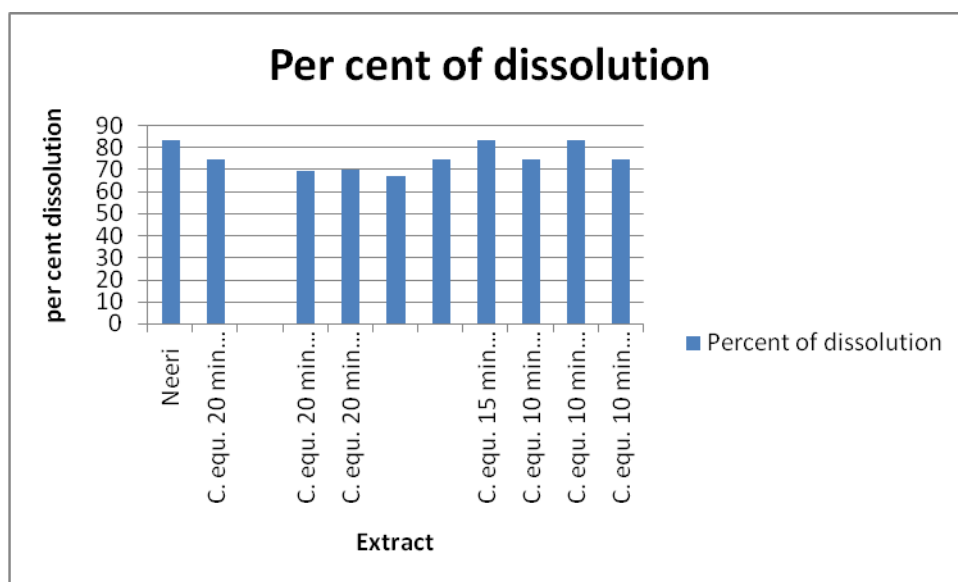


Fig. 3: Percentage dissolution of calcium oxalate by Gamma irradiated *Casuarina equisetifolia* extract groups.

in the same place for a period of time equal to that for irradiated eggs. Control eggs were subjected to room temperature. After 10, 15 and 20 min, egg shells were kept at a temperature of 35°C (Fig. 3). Irradiated eggs shells and egg shells from non-irradiated eggs were kept under the same conditions (Vilic *et al.*, 2010).

Transmission Electron Microscopy:

Transmission Electron Microscopy (TEM) is a technique that uses an electron beam to image a nanoparticle sample, providing much higher resolution than is possible with light-based imaging techniques. TEM is the preferred method to directly measure nanoparticle size, grain size, size distribution, and morphology. The transmission electron microscope (TEM) has evolved over many years into a highly sophisticated instrument that has found widespread application across many scientific disciplines. Because the TEM has an unparalleled ability to provide structural and chemical information over a range of length scales down to the level of atomic dimensions, it has developed into an indispensable tool for scientists who are interested in understanding the properties of nanostructured materials and manipulating their

behavior.

The resolution of the optical microscope is restricted by the wavelength of visible light, which thus precludes atomic-scale imaging (David, 2015).

Preparation of calcium oxalate crystals:

By taking equimolar solution of calcium chloride dihydrate dissolved in distilled water and sodium oxalate was dissolved in 10 ml of 2N H₂SO₄, sufficient quantity is allowed to react in a beaker. The resulting precipitate of calcium oxalate which was freed from traces of sulphuric acid by washing with ammonia solution (Fig. 4a). Then again it was washed with distilled water and dried at a temperature of 60 °C for 4 h (Moe, 2006).

Preparation of the Semi permeable membrane from farm eggs:

The outer calcified shell was removed chemically by placing the eggs in 2 ml HCl for overnight, which caused complete decalcification. Further, washed with distilled water and carefully with a sharp pointer a hole is made on the top and the contents squeezed out completely from the decalcified egg (Figs. 4 b, 5). It was then washed thoroughly with distilled water and placed it in ammonia solution, in the moistened condition for

a while and then rinsed it with distilled water. Stored in refrigerator at a pH of 7- 7.4 (Jha *et al.*, 2016).

Titrimetry method:

1 g of crude sample was dissolved in distilled water, ethanol and methanol. Then it was treated with Ultrasonication with different probes (probe 3, 5, and 10). Weighed exactly 1 mg of the calcium oxalate and 10 mg of ethanolic extract, water extract, methanolic extract and standard Neeri were packed in semi permeable membrane by suturing as shown in Model design . They were allowed to suspend in a conical flask containing 100 ml 0.1 M Tris buffer. One group served as negative control (contained only 1 mg of calcium oxalate). Conical flasks of all groups were placed in an incubator pre-heated to 37 °C for about 7-8 h. Two sets are placed for this i.e. one set is treated with Gamma radiation and one set is normal. Contents of semi permeable membrane from each group was removed into a test tube. Added 2 ml of 1 N sulfuric acid and titrated with 0.9494 N KMnO₄ till a light pink color end point obtained. 1 ml of 0.9494 N KMnO₄ equivalent to 0.1898 mg of calcium oxalate.

The amount of calcium oxalate that was dissolved was subtracted from the total quantity of calcium oxalate used in the experiment. This showed the actual quantity of calcium oxalate the test drug can dissolve. In dissolution study the negative control showed zero dissolution. The standard group (Neeri) showed dissolution of 83.7%. The aqueous extract and the alcohol extract of test drug (*C. equisetifolia*) showed dissolution of 66.96% and 83.7%, respectively. Except standard group the aqueous extract of test

drug (*C. equisetifolia*) showed maximum dissolution of 82.7% (Saso *et al.*, 1998).

Turbidity method:

Turbidometric method measures the turbidity in terms of calcium oxalate formation in synthetic urine using spectrophotometer at 620 nm and crystallization inhibition measured by turbidity reduction. Stone nucleus was grown *in vitro* in the absence of any inhibitor. (Khan *et al.*, 1992)

Results and Discussion

Urolithiasis is a common painful disease, which afflict human population since ancient times. Those composed of calcium oxalate are the most common uroliths accounting for more than 80% of the stones. The mechanisms involved in the formation of calcific stones are not fully understood but it is generally agreed that urolithiasis is a multifaceted process involving events leading to crystal nucleation, aggregation and growth of insoluble particles. Various therapies like diuretics are being used in attempt to prevent recurrence of hypercalciuria and hyperoxaluria induced calculi but scientific evidence for their efficacy is less convincing. Medicinal plants have played a significant role in various ancient traditional systems of medication. Even today, plants provide a cheap source of drugs for majority of world's population. Several pharmacological investigations on the medicinal plants used in traditional antiurolithic therapy have revealed their therapeutic potential in the *in vitro* models.

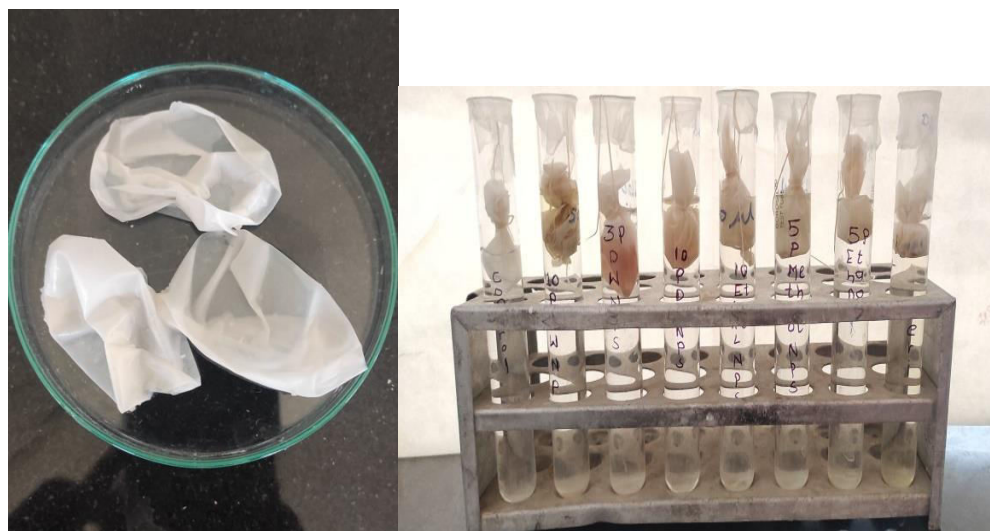
The *in vitro* antiurolithiatic study of the whole plant of *Casuarina equisetifolia* through titrimetric and turbidity method has showed extremely

Table 3: Results of turbidity method by plant extract *Casuarina equisetifolia*

Control	Turbidity Neeri	Turbidity in 3 probe distilled water	Turbidity in 3 probe methanol	Turbidity in 3 probe ethanol	Turbidity in 5 probe distilled water	Turbidity in 5 probe methanol	Turbidity in 5 probe ethanol	Turbidity in 10 probe distilled water	Turbidity in 10 probe methanol	Turbidity in 10 probe ethanol
0.14	0.83	0.01	0.61	0.01	0.01	0.06	0.08	0.06	0.34	0.09



(a) (b)
Fig. 4: (a) Calcium oxalate crystals and (b) decalcification of egg shell in 10% HCl.



(5)
Fig. 5: Egg shell membrane
Std (Neeri), (semipermeable).

(6)
Fig. 6: The calcium oxalate stone with Control,
aqueous extract and alcoholic extract.

significant action on urinary calculi. Titrimetric estimation measures undissolved calcium oxalate by using KMnO_4 . The Gamma irradiated aqueous extract of 20 min, 15 min and 10 min of test drug showed dissolution of 74.89%, 66.96% and 74.89%, respectively. The Gamma irradiated ethanolic extract of 20 min, 15 min and 10 min of test drug showed dissolution of 70.11%, 83.7%,

74.89%, respectively. The Gamma irradiated aqueous extract of 20 min, 15 min and 10 min of test drug showed dissolution of 69.69%, 74.89%, 83.7%, respectively. In case of ultrasonication samples 3 htz, 5 htz, 10 htz aqueous extract showed dissolution of 74.89%, 66.96%, 66.96%, respectively. Samples 3 htz, 5 htz, 10 htz of methanolic extract showed equal or same

dissolution of 83.7%. Samples 3 htz, 5 htz, 10 htz of ethanolic extract showed dissolution of 83.7%, 83.7%, 74.89%, respectively which was significant compared to standard group (Neeri 83.7%). Turbidometric method measures the turbidity in terms of calcium oxalate formation in synthetic urine using spectrophotometer at 620 nm and crystallization inhibition measured by turbidity reduction. The turbidity showed by the alcohol extract and the aqueous extract of test drug (*C. equisetifolia*) was highly significant compared to the standard drug.

Turbidometric method measures the turbidity in terms of calcium oxalate formation in synthetic urine using spectrophotometer at 620 nm and crystallization inhibition measured by turbidity reduction. The turbidity showed by the alcohol extract and the aqueous extract of test drug (*C. equisetifolia*) was highly significant compared to the standard drug (Table 3). There are many other plants which has been studied and tested for antiurolithiatic activity such as *Achyranthes aspera*, *Lawsonia inermis*, *Ficus benghalensis*, *Raphnus sativus*, *Macrotyloma uniflorum* and *Scoparia dulcis* Linn. Among these *Scoparia dulcis* Linn. showed higher dissolution percentage of 66.96%. Compared to all these plants *Casuarina equisetifolia* showed higher percentage dissolution of calcium which equal to std. group Neeri. The result of the present study showed that the selected plant possess antiurolithiatic activity, which was directly proportional to the concentration of the extract. These findings substantiate the traditional use of the plants in the treatment of urinary stones and kidney problems.

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