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ANTIUROLITHIATIC ACTIVITY OF NEEM LEAVES IN EXISTING RENAL CALCULI BY *INVITRO* METHODS.

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ABSTRACT
Kidney stones or calculi are most prevalent diseases around the world. Calcium oxalate was found to be the most important component of the calculi. Most of the ayurvedic practitioners use ethanobotanical herbs to cure these calculi. But as such no such scientific evidence is available till date on these plants. The present study was focussed mainly to study the inhibitory effects of the *A.indica* (Neem plant) on the stone formation. Neem leaves ash was used to demonstrate the invitro activity on the stone formation. Leaves ash was pulverized and was extracted with solvents like Methanol, Chloroform and Water. The invitro activity was performed to estimate the inhibitory activity of these extracts on the formation of calculi. NEERI and Cystone were used as positive control. Antiurolithiatic activity was studied by titrimetry and turbidity methods. Methanol extract of sample was proved to competent enough with the standard drugs, cystone and NEERI in dissolving the existing calcium oxalate stones. Methanol, Chloroform and water extracts showed 53%, 38% and 32% of dissolution of stones respectively. And Cystone and NEERI showed 65% and 62% respectively. Maximum inhibition in calculi formation (turbidity) was seen by the methanol extract after 210 seconds of chemical reaction as compared to the control, Cystone and NEERI. Chloroform and water extracts both showed similar activity, but still chloroform extract being the second. At present Cystone and NEERI were prescribed by medical practioners as effective medicine for preventing and treating the calculi formation. The present study confirms of the activity of our sample in comparison to the standard drugs. Further we plan to study the properties in detail and try to optimize the parameters to make it more efficient in treating the calculi.

KEYWORD: Calculi, Calcium oxalate, Turbidity method, Neem leaves, Cystone.

INTRODUCTION
Many traditional and folklore remedies are available for the diseases which are still waiting for treatments. The ethanobotanical herbs do contain many important phytochemical compounds such as tannins, alkaloids, carbohydrates, flavonoids and phenols. These bioactive substances provide a definite physiological action on the human body [Edogo HO, 2005; Mann J. 1978]. These compounds are usually the byproducts produced during the primary or secondary metabolism of organism. The whole or part of the plant can be used in the phytomedicine. The phytochemical constituents can be extracted from the leaves, fruits, flowers or inflorescence and seeds [Criagg GM, 2001; Adriano R, 2000].

Urolithiasis is becoming the most common global problem affecting the human beings [Cowan MM, 1999]. Kidney calculi disease is now considered as a common chronic disorder in humans and the commonly occurring renal stone is made up of calcium oxalate [Butt AJ. 1956]. Calcium stone formation occurs in several stages including nucleation, growth of the crystal, aggregation of the crystalline bodies and crystal retention [Unnati Atodariya, 2013]. Kidney stone formation is the result of a physicochemical process that involves nucleation of crystals from a supersaturated solution [Charles C Thomas, 2007]. The most common components of kidney stones are calcium, oxalate and phosphate. The factors that influence the formation of crystals are urine volume and concentration of constituents of the stone and several other factors which might inhibit or promote stone formation [Vasu K, 2009].

In spite of fast emerging treatment and diagnosis of the urolithiasis, still there is no such medicine available so far to use in the clinical therapy [Cox PA, 1994]. Ethanobotanical herbs promises the pin point accuracy in treating the acute and chronic urolithiasis [Neube NS, 2009]. Ethanomedicine normally uses the plants and natural environment for healing purpose. Most of the pharmaceutical companies depend on these plants in search of novel drugs [Jain Monika, 2012].

In the present study, we aimed to claimed the activity of the extract to be effective in the treatment of kidney stone formation. *Azadirachta indica* is well known in India and its neighbouring countries for more than 2000 years as one of the most versatile medicinal plants having a wide spectrum of biological activity. Neem has been extensively used in ayurveda, unani and homoeopathic medicine. Neem elaborates a vast array of biologically active compounds that are chemically diverse and structurally complex [R. Subapriya, S Nagini, 2004]. It is now considered as a valuable source of unique natural products for development of medicines against various diseases and also for the development of novel industrial products.

Keeping above knowledge in the mind, current study is done to find out the stone formation inhibitor effect and stone dissolving effect of *A. indica* extract.

MATERIAL AND METHOD

Plant material collection and extract preparation: *A.indica* leaves were collected and washed thoroughly to remove the dust particles. They are then burnt into ash and grinded mechanically. Powdered material is then used for the extraction process using Methanol, Chloroform and Water. All the extracts were collected and filtered with whatman filter paper and evaporated in a rotary evaporator at 65^0C. The powdered sample is then weighed and used for further experimentation. For the control treatment, herbal formulation Cystone and NEERI are used and their stock solution are prepared by suspending them in DMSO solution (50mg/ml) and filtered through whattman filter paper. The aqueous extract of Cystone was prepared by grinding a tablet to powder. This powder was mixed with 5ml water and kept for 2–3 h and then centrifuged at 1000 rpm. The clear supernatant was used for the study.

Evaluation for Anti-urolithiatic Activity: (Byahatti V, 2010)

Production of kidney stones

Experimental kidney stones or Calcium oxalate stones were prepared by homogenous precipitation method described by Byahatti et al, 2010. Equimolar solution of Calcium chloride dihydrate in distilled water and Sodium oxalate in 2N H2SO4 were both allowed to react in saturated amounts of distilled water. The precipitate obtained was calcium oxalate. Similarly, equimolar solution of Calcium chloride dehydrates in distilled water and Disodium hydrogen phosphate in 2N H2SO4, was allowed to react in sufficient quantity of distilled water. The resulting precipitate was calcium phosphate. Both precipitates were washed with excess ammonia solution to free them from traces of sulphuric acid. The precipitates obtained were then washed thoroughly with distilled water and dried at 60^0C for about 4 -6 hours.

Egg membrane Preparation

Chicken eggs were used for the experiment. The semi - permeable membrane usually lies between the outer calcified shell and the inner albumin & yolk. Shell was removed chemically by placing the soaking the eggs in 2M HCl overnight. After the complete decalcification of the eggs they were further washed with distilled water. The contents were then squeezed out completely by carefully puncturing a hole on the top. Then membrane obtained was washed thoroughly with distilled water, and placed in ammonia solution for deacidification. The membranes were further stored at 4^0C until further use.

Estimation of Calcium oxalate by Titrimetry

About 2mg of the calciumoxalate and 20mg of the extract compound was packed in the semi permeable membrane. The membrane was sutured at one end and placed in a conical flask containing 100ml of 0.1M Tris buffer. Calcium oxalate without the extract being the negative control. Cystone and NEERI were used as positive control. The conical flasks were then incubated in a preincubated chamber at 37^0C for about 8 hours. Following incubation the contents from the sac were collected into a test tube. To the contents 2ml of 1N sulphuric acid was added and titrated against the 0.94N $KMnO_4$ till the appearance of light pink colour. 1ml of 0.9494N $KMnO_4$ equivalent to 0.1898mg of Calcium. The percent dissolution of the calcium oxalate was calculated using the amount of undissolved calcium oxalate.

Anti-lithiatic activity test by turbidity method (Bensatal A; Ouahrani M R, 2008)

In vitro anti-lithiatic activity of the extracts were demonstrated in terms of inhibition of calcium oxalate formation by the extracts in the presence and absence of inhibitors (standard drug and extract). The precipitation of calcium oxalate at 37^0C and pH 6.8 has been studied by the measurement of turbidity at 620nm. To 1ml of 0.025M $CaCl_2$ 2ml of Tris buffer (pH 7.4) was added and mixed thoroughly. To this 1ml of 0.025M of sodium oxalate is added and the turbidity formed is measured at 620nm. This serves as negative control. The experiment is repeated with the standard drugs (Cystone and NEERI) and the sample extracts (50mg/ml). Standard drugs act as positive control. The percent of inhibition of the drugs and the standard was measured until a period of 10min. the percent of inhibition is then calculated using the formula Inhibition % = {1-(Si / Sc)x100. Where; Si is slope of graph in the presence of inhibitor (drug / extract) and Sc is slope of graph without inhibitor (control).

RESULTS AND DISCUSSION

Estimation of Calcium oxalate by Titrimetry

The titrimetry values obtained clearly showed the urolithiatic activity of the extracts. The methanol extract showed highest activity when compared to the other two extracts (Chloroform and Water). The % dissolution was found to be 53±0.232, 38±0.113 and 32±0.334 for Methanol, Chloroform and Water extracts respectively. Standard drugs like cystone and NEERI showed 65±0.043 and 62±0.024 respectively. Methanol extract showed higher activity in par with the standard drugs.

Table 1: Percent Dissolution of Calcium oxalate by the extracts. Cystone and NEERI being the positive control. All the values are average of triplicates. The percent dissolution was expressed as % ± se.

	Vol of Std KMnO4 (ml)	Wt of Calcium estimated	Wt of Calcium reduced	% Dissolution
NEERI	1.3	0.247	0.398	62±0.024%
Cystone	1.2	0.228	0.417	65±0.043%
Methanol	1.8	0.304	0.341	53±0.232%
Chloroform	2.1	0.399	0.246	38±0.113%
Water	2.3	0.437	0.208	32±0.334%
Negative	3.4	0.645		

Anti-lithiatic activity test by turbidity method

The turbidity studies done on the extracts revealed of the inhibitory activity in the stone formation. Maximum inhibition was formed by the methanol extract (58%) after 210seconds. Chloroform (42%) being the second and water extract (41.23%) being the third. Chloroform and water extract were both similar in their activity when compared to the methanol extract. Two control drugs NEERI and Cystone inhibited 80.28 and 81.24% in stone nucleation respectively. A two way ANOVA between the extracts and nucleation formation at different time intervals was conducted to compare the effect of extracts on the nucleation in stone formation. There was a significant effect of different extracts and time period in seconds remembered at the $p<0.05$ level. The significance effect of the extracts on the stone nucleation formation [$F(5,60) = 108.234$, $p = 1.06E-28$] and [$F(12,60)=55.314$, $p=7.7E-05$].

Table 2: Table showing the Absorbance observed for the plant extracts and the control drugs after 210sec. All the values were average of triplicates.

Treatment groups	Absorbance at 620nm after 210sec
NEERI	0.45
Cystone	0.53
Methanol	0.62
Chloroform	0.73
Water	0.73
Negative	1.03

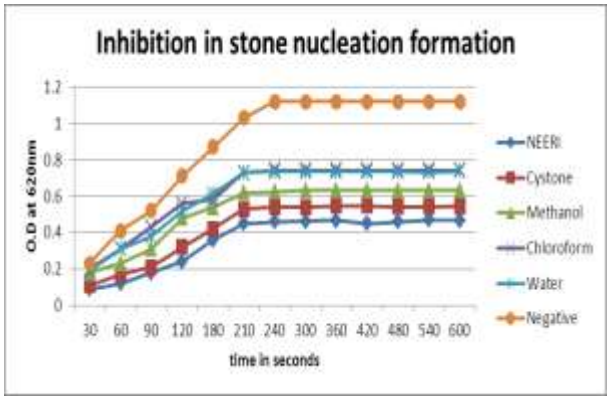

Fig 1: Graph showing the inhibition in the stone nucleation formation with and without plant extract. Cystone and NEERI being the positive controls. All the values were average of triplicates.

DISCUSSION

Ethanobotanical herbs are getting a great deal of attention for their medicinal properties. Plant drugs are receiving a great concern than the chemically synthesized drugs as they have minimum side effects and greater efficacy. Though the results seemingly were good, the plant based crude extracts were literally complex compounds of two or more phytochemicals. Its practically tiresome to find out which component is doing the healing.

In our study the sample extracted with methanol fraction was found to show the inhibition in the crystal stone formation. This confirms of the antilithiolytic activity of the plant against the calculi. As discussed earlier, the kidney stone formation is complex mechanism involving several physic chemical process and to avoid such stone nucleation process several drugs were used. Cystone and NEERI were used as positive controls in our experiment, which are widely used by both allopathic and ayurvedic physicians. Our experimental plants have been proved of their capacity to inhibit the formation of stone nucleus and further we plan to isolate and purify the compound to find out the component which actually does the activity.

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