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"EFFECT OF HOMOEOPATHIC MEDICINE TRIBULUS TERRESTRIS Q, 6C, 12C, 30C, 200C, 1M AS AN INHIBITOR OF CALCIUM OXALATE AND CALCIUM PHOSPHATE CRYSTALLIZATION *IN VITRO* STUDY"

Homeopathic

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ABSTRACT

Background: Urolithiasis is one of the common conditions in the society and it needs medical attention due to its increase in prevalence. The use of the homeopathic medicines has found to be,of great importance in the treatment of urolithiasis and certainly homoeopathy is a promising field in this condition.

Objectives: The aim of this study was to evaluate the mechanism of urolithiasis and the inhibitory action of homeopathic drug Tribulus terrestris by *in vitro* experiment.

Materials and Method: Homoeopathic preparation of Tribulus terrestris Q, 6C, 12C, 30C, 200C, 1M was planned to evaluate *in vitro* calcium oxalate and calcium phosphate crystallization using spectro-photometric and colorimetric assay respectively. Considering the role of reactive oxygen species as one of the etiological factors in stone formation, effective antioxidant activity of Tribulus terrestris was also performed by 2,2-diphenyl -1-picrylhydrazyl (DPPH) free radical scavenging assay.

Result: Tribulus terrestris Q, 200C and 1M exerted maximum inhibition as 33.62%, 23.89% and 23.00% respectively to calcium oxalate nucleation assay whereas, Tribulus terrestris Q, 6C, 12C, 30C, 200C, 1M exerted maximum inhibition to calcium oxalate aggregation assay up to 76.19%. Tribulus terrestris 12C and 30C showed maximum inhibition as 82.28% and 16. 21% to calcium and phosphate ions respectively. Presence of antioxidant activity by DPPH radical assay for Tribulus terrestris Q and 12C which showed percentage inhibition as 33.11% and 0.95% respectively.

Conclusions: Homoeopathic preparation Tribulus terrestris has potential effect on inhibition of calcium oxalate and calcium phosphate crystallization and also homoeopathic preparation of Tribulus terrestris is capable of showing presence of phytochemicals; anti-oxidant activity when performed by in vitro experiment.

KEYWORDS

Urolithiasis; Crystallization; Inhibition; Homoeopathy; Tribulus terrestris.

INTRODUCTION -

Science demands an unfolding of the mystery that intrigues the inquisitive, the research minded. This unfolding in the time-space dimension is an essential part of the scientific development. Homoeopathy, being a science, is not an exception to this. Homoeopathic system is unique and different as compared to other systems of health sciences. It is a system of drug therapeutics based on the Law of Similar. This law states that a substance which can produce symptoms in a healthy individual is also capable of curing the same in sick individual. To verify this idea some experimental studies are going on such as *in vitro* or *in vivo* studies where artificially induced diseases or medical condition for instance, Candidiasis or urolithiasis are tested by using homoeopathic medicines which areable to alter the Patho-physiological functions by inhibitory process in above mentioned medical conditions.

Homoeopathic medicines are prepared from an extensive range of natural sources. In other words, ubiquitous sources of homoeopathic Materia medica areMother Nature itself. Over around 75% of the medicines in homoeopathy are prepared from the vegetable kingdom where various parts are used for the preparation of the homoeopathic medicines in potentised form [1].Ayurveda and other system of Indian Medicine, uses mostly the medicines prepared from vegetable kingdom but in crude form.Homoeopathic Materia Medica has now come a long way through the investigatory process initiated by Master Hahnemann. While bringing to the fore the neglected relationship between a drug and the disease, through the formulation of the Law of Similar, Hahnemann placed before the profession the solution, which nature has so kindly bestowed upon all of us.

Due to variations in socio-economic statusandgeographic locations, the incidence and prevalence of urolithiasis has changed in different countries over a period. The review says the prevalence of urolithiasis is about 5% to 19.1%inWestAsia,SoutheastAsia,SouthAsia. Interestingly, it is only 1% to 8% in most part of East Asia and North Asia and 5-7% in India (especially South public are more prone than North). The recurrence raterangesfrom21%to53%after3-5years [2]. Highest incidence of kidney stonesis observed in 30-45 years age group and decreases after the age of 50 years. Specifically, in India it occupies some parts of Maharashtra, Gujarat, Punjab, Haryana, Delhi and Rajasthan. Urinary stones continue to dwell in everyday urological practice. In Manipur, the situation is not an exception as the incidence of urolithiasis is high. From hospital records for a period of seven years and three months, urolithiasis cases were observed to be 11.6% [3]. The prevalence of urolithiasis is as high as 7.6% in Satpura part of Maharashtra. Around 12.7% of India's population depends solely on homoeopathy for their health care [3]. Apparently, Homoeopathy has been proved to be a boon for the patients in whom surgery is a risky affair such as aged ones, hypertensive and diabetics or those who are in search of an alternative to surgery. Homoeopathy recognizes human as a multi-dimensional compound entity where mind, body and spirit are seen as a union and indivisible.

Types of kidney stones

Urinary stones areaggregates consisting of crystals and organic matrix. Stone matrix accounts for 2-3%, various macromolecules like proteins (64%), non-amino sugars (9.6%), hexosamine as glucosamine (5%), bound water (10%) and rest as bound ash (Boyce, 1968). Lipid has also an important component of stone matrix [4].

Calcium stones: CaOx dihydrate, CaOx monohydrate, Calcium phosphate [5].

Non-calcium stones: Magnesium ammonium phosphate, carbonate apatite, matrix calculi [5].

Uric acid and Urates: Uric acid, Ammonium urate, sodium urate [5].

Cystine stones: Genetic disorder is the major cause for the formation of cystine stones [5].

Drugs: Indinavir, Triamterene [5].

Mechanism of Stone Formation

Super saturation

Initial phase of stone formation takes place bytubular fluid supersaturation which commonly occurs in the loop of Henle and it provides thepowerful force for the development of interstitial calcium crystals deposits known as Randall's plaques. The deposition of crystals occurs in interstitial sites in the inner medulla. Based on the concentration of crystallizable salts, there are three major states of saturation of urine. 1. Under saturated i.e., concentration of crystallizable salts less than the solubility product, 2. Metastable i.e., concentration between solubility product and the formation product, 3. Unstable i.e., concentration greater than the formation product [5,6].

Nucleation

The step in the transformation from a liquid to a solid phase in which free ions in solution associate into micro-particles in a supersaturated solution is called nucleation. Nuclei form the first crystals that do not dissolve and have a characteristic lattice pattern.Epithelial cells, urinary casts, RBCs, cell debris and other crystals can act as nucleating centres in urine (Smith, 1990). Renal tubular cell injury can promote crystallization of CaOx crystals by providing materials for their heterogeneous nucleation [5,6].

Crystal growth

Crystal growth is one of the pre-requisites for particle formation and thus for stone formation. Once a crystal nucleus has achieved a critical size and relative supersaturation remains as it is. Growth of micro-crystals is accomplished by movement of ions out of solution onto the growing crystal. It may happen that the growth of these micro-crystals to the extent that they can be retained in the kidney on the basis of size alone without aggregation[5,6].

Aggregation

Aggregation is a process in which there is accumulation of crystals that form in free solution into larger multi-component particles.In stone formation the aggregation of crystals and an organic matrix; proteins, lipids, polysaccharides, and other cellderived material. serves as the binding mediator. Cell membranes and their lipids play critical roles in the process of calcification.The process of growth is so slow that crystals cannot become large enough to obstruct the renal tubules and be retained there by this mechanism alone [5,6].

MATERIALS AND METHODS -

Drugs and Chemicals

All the chemicals were of analytical grade and procured from SRL (Sisco Research Laboratories Pvt. Ltd.). Homoeopathic medicines which are manufactured by standard Homoeopathic Pharmacy; as per the norms of Homoeopathic Pharmacopoeia India. Tribulus terrestris Q, 6C, 12C, 30C, 200C, 1M was made available from SBL (Sharda Boiron Laboratories Ltd.), Ethyl alcohol (as per HPI strengths) was used as vehicle control. Equipment used were JASCO-UV/VISIBLE-630 Spectrophotometer, quartz cuvette, Digital Photo-Colorimeter, Centrifuge 5424 R Machine.

Calcium Oxalate (CaOx) crystallization assay

CaOx crystallization assay was carried out using *Hess et al* method with few modifications [10]. Stock solutions were prepared of $CaCl_2$ (8.5 mM) and $K_2C_20_4$ (1.0 mM), containing 200 mM NaCI and 10 mM sodium acetate, pH was adjusted to 5.70. Further, 1.0 ml of the $CaCl_2$ is transferred into quartz cuvette.0.1ml of Tribulus terrestris containing Q, 6C, 12C, 30C, 200C, 1M and ethanol as a control were added to each solution of $CaCl_2$ respectively. Finally, 1.0 ml of the $K_2C_2O_4$ solution was added to light path quartz cuvette and was placed in a spectrophotometer at 37ºC,at fixed OD (Optical Density)620nm [10].Calcium Oxalate crystallization assay was carried out in which slope of nucleation till maximum and slope of aggregation after the peak were recorded for every 60 seconds for 20 minutes.The assay was performed in triplicates for each potency of Tribulus terrestris and control [9,10].

Percentage Inhibition for Nucleation assay = (SNc-SNt/SNc) x 100

Percentage Inhibition for Aggregation assay = (SAc-SAt/SAc) x 100

Where;SNc is slope of nucleation for control

SNt is slope of nucleation for test

SAc is slope of aggregation for control

SAt is slope of aggregation for test

Calcium Phosphate (CaP) assay

To demonstrate the amount of Calcium Phosphate precipitation, homogenous Crystallization system was used to study the mineral phase formation by *in vitro*. (Chaudhary et al 2010)[8].

Initially 5.0 ml system was prepared containing 2.5ml of Tris Buffer (pH 7.4), 0.5ml of 50 mM $CaCl_2$ and 0.5ml of 50 mM KH_2PO_4 with 0.1ml Tribulus terrestris Q, 6C, 12C, 30C, 200C 1M and ethanol as control. After incubating this assay system at 37ºC, precipitates obtained were centrifuged at 4500 rpm for 15minutes and precipitateswere dissolved in 5ml of 0.1 N HCl. The calcium ions (Ca^{2+}) and phosphate ions (HPO_4^{2-}) concentration in the precipitate represents the degree of precipitation of these ions and the sample containing Tribulus terrestris minimized the extent of their precipitation [8].

The calcium and Phosphate ions were estimated by Trinder, 1960 and Gomeri,1941 method respectively [11,12].

DPPH free radical scavenging assay

The scavenging activity of homoeopathic dilution of Tribulus terristris for the potency having highest inhibition and aggregation in both CaOx and CaP assay is Q and 12C respectively was checked with DPPH radical.[14] A spectrophotometric assay was used for determination of scavenging activity of Tribulus terrestris [13]. A volume of 0.1 mM solution of DPPH was prepared by adding 25 mg of DPPH in 100 ml of methanol. A volume of 90 µl solution of DPPH and different serial dilutions of 10 ul Tribulus terristris Q and 12C was placed in light path quartz cuvette of Spectrophotometer. The DPPH and 10 µl of ethanol were used as negative control.The whole process was done in triplicate. The absorbance was measured at 517 nm in spectrophotometer [13,14,15].

Scavenging activity % = ([Ac − At)/Ac] × 100

Where;Ac: Absorbance of control

At.: Absorbance of test

Absorbance reflects the amount of anti-oxidant activity present in Tribulus terrestris.

STATISTICALANALYSIS

All the data found in the study is subjected to analysis of variance using one way ANOVA and linear regression analysis wherever necessary. Graphpad-Prism version 9 for graphical presentation.

Results and observations

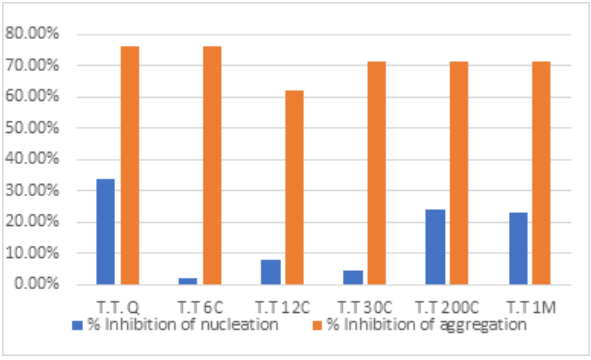
Inhibition of Tribulus terrestris on CaOx crystallization

Table No. 1 shows effect of different potencies of Tribulus terrestris. In CaOx nucleation the highest percentage inhibition was 33.62% exerted by Mother tincture. There was constant decrease in percentage inhibition by 6C,12C ,30C as 1.76%,7.96%, 4.42% respectively as the potency increased. But 200C and 1M exerted moderate inhibition i.e., 23.89% and 23.00% respectively as compared to lower potencies.

In CaOx aggregation process all tested potencies of Tribulus terrestris showed good inhibitory process. Highest percentage inhibition of aggregation was exerted by mother tincture and 6C as 76.19%. The inhibitory process of aggregation was slightly decreased in 12C as 61.90% then there was constant increase in percentage of inhibition from 30C, 200c and 1M as 71.42%.

Table No.1 - Percentage inhibition of Tribulus terrestris on CaOx crystallization by nucleation and aggregation assay

Test	% Inhibition of nucleation	% Inhibition of aggregation
Tribulus terrestris Q	33.62%	76.19%
Tribulus terrestris 6C	1.76%	76.19%
Tribulus terrestris 12C	7.96%	61.90%
Tribulus terrestris 30C	4.42%	71.42%
Tribulus terrestris 200C	23.89%	71.42%
Tribulus terrestris 1M	23.00%	71.42%



Graph No. 1- Inhibition of CaOx crystallization

Inhibition of Tribulus terrestris on CaP crystallization

Table No. 2 shows percentage inhibition exerted by different potencies of Tribulus terrestris on Calcium ions with significant variations in all potencies. Amongst all 12C of Tribulus terrestris showed the maximum inhibition as 82.28 %.

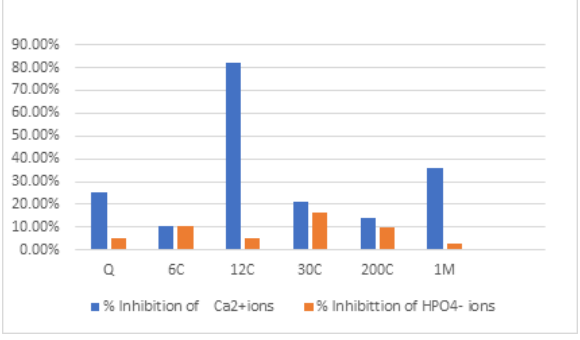
Table No. 2- Effect of Tribulus terrestris on Ca2+ions crystallization

Test	Absorbance of Ca^{2+} ions	Concentration of Ca^{2+} ions	% Inhibition of Ca^{2+} ions
Control	0.28	17.50	--
Tribulus terrestris Q	0.21	13.12	25.02%
Tribulus terrestris 6C	0.25	15.62	10.74%
Tribulus terrestris 12C	0.05	03.10	82.28%
Tribulus terrestris 30C	0.22	13.75	21.42%
Tribulus terrestris 200C	0.24	15.00	14.28%
Tribulus terrestris 1M	0.18	11.25	35.71%

Table No. 3 shows percentage inhibition exerted by different potencies of Tribulus terrestris on HPO4- ions which was constant through all potencies with insignificant variations. The maximum inhibition was exerted by 30C as 16.21%.

Table No.3- Effect of Tribulus terrestris on HPO4- ions crystallization

Test	Absorbance of HPO^{4-} ions	Concentration of HPO^{4-} ions	% Inhibition of HPO^{4-} ions
Control	0.37	23.87	--
Tribulus terrestris Q	0.35	22.58	05.40%
Tribulus terrestris 6C	0.33	21.29	10.80%
Tribulus terrestris 12C	0.35	22.58	05.40%
Tribulus terrestris 30C	0.31	20.00	16.21%
Tribulus terrestris 200C	0.33	21.29	10.08%
Tribulus terrestris 1M	0.36	23.22	02.72%



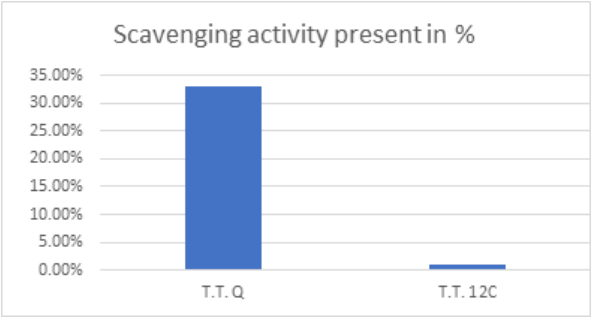
Graph No. 2- Inhibition of CaOx crystallization on Calcium and Phosphate ions

Effect of Tribulus terrestris on anti-oxidant property

Considering the objective of this study scavenging activity was performed for mother tincture and 12C of Tribulus terrestris to test the presence of anti-oxidative property. Anti-oxidant activity plays a major role as a reactive oxygen species in reduction of oxidative stress which helps in inhibition of urolithiasis. In this study mother tincture and 12C showed presence of anti-oxidant property as 33.11% and 0.95% respectively.

Table No. 4- Scavenging activity of T.T. to the potencies having maximum inhibition

Test	Absorbance	Scavenging activity present in %
Control	0.7746	–
Tribulus terrestris Q	0.5181	33.11%
Tribulus terrestris12C	0.7672	0.95%



Graph No. 3- Scavenging activity of Tribulus terrestris in percentage inhibition

DISCUSSION -

The preparation of homeopathic medicines was first invented by father of homoeopathy Dr. Samuel Hahnemann. The Principle/Doctrine of Drug Dynamization which is one of the cardinal principles of homoeopathyintroduced in the publication of Organon of Medicine, fifth edition, in 1833 [16]. It states about preparation of Homoeopathic drugsin the desired potencies. Homeopathic medicines are micro-dosed natural substances derived from plant, animal or mineral sources. A specific homeopathic medicine dilution is attained by an exact and meticulous process of successive 'homeopathic dilution". This process transforms the original substance into a therapeutically active medicine.It is claimed that the lower the concentration of a substance, the more potent it becomes. This concept, often referred to as the "Law of Infinitesimals," [16]. The action of homeopathic medicine is a debatable question where many studies, theories, experiments have been undertaken to explain the molecular mechanism of homoeopathy through quantum theory, water molecule theory etc. but still the exact mechanism is unknown to the world. Homeopathic drugs are capable of acting on functional and structural level when administered in dilution form. They act on vital organs so deep that they can alter the cellular structure. In the mechanism of urolithiasis i.e., nucleation, aggregation, growth process, homoeopathic medicine *Tribulus terrestris* was able to altered the course of its mechanism. The inhibitory action of *Tribulus terrestris* is may be due to the presence of active principles like saponins, flavonoids, glycosides, alkaloids, and tannins [17]. To understand presence of active principles in Homoeopathic preparation of *Tribulus terrestris* further study is required. Also, a study proposes that the homoeopathic drug possessing antioxidant property is capable of reducing oxidative stress which results in reduction of renal damage which is one of the major mediators in patho-physiology of urolithiasis [18] hence presence of anti-oxidant property in *Tribulus terrestris* proves to be helpful in inhibition of urolithiasis.

To prove homoeopathy as a science more advanced studies are required in the field of homoeopathy other than clinical trials. There are very less in vitro and in vivo studies in homeopathy against urolithiasis which should be performed in the laboratory and also for the phyto-chemical actions of the homoeopathic drugs. Such type of study will be highly useful for performing research in the field of homeopathy and it will open up a new vista of understanding to perceive the action of homoeopathic drugs when given in potentized form. This will create a platform to compare the use of homeopathic, Ayurvedic and Allopathic drugs in their respective preparation bases.

CONCLUSION -

Based on aboveresults, we can conclude that Homoeopathic medicine Tribulus terrestris Q, 6C, 12C, 30C, 200C, 1M has effect on inhibition

of Calcium oxalate and Calcium phosphate Crystallization when performed by in vitro study. To state further mother tincture and lower potencies can give considerable effects when administered clinically.

Homoeopathic medicine Tribulus terrestris also possess anti-oxidant property which indicates that evenwhenhomeopathic medicines prepared in potentized formthey preservetheir therapeutic properties as potential.

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